Inflammation is not a contributing factor in the development of insulin resistance in diet induced obesity in rats

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Medical Research)

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University of Tasmania

April 2017

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ACKNOWLEDGEMENTS

First of all I would like to acknowledge and thank my supervisors Dr. Stephen Richards, Prof. Stephen Rattigan, and Dr. Michelle Keske for their help and guidance with this project and thesis.

Many thanks go to past and present members of the Muscle Research Group who have given their time and friendship over the years. I would especially like to thank Eloise Bradley, Dr. Dino Premilovac, and Dr. Helena Ng who taught me many of the laboratory techniques and who always gave their time to answer questions and come to my aid when I needed help or advice. Eloise and Dino require additional thanks for the high fat and metformin chapters – you saved my butt yet again and I am forever grateful. I would also like to thank Renee Dwyer who always had a smile, always tried have something positive to say (even if things were not great), and was always interested in our personal lives and keeping us (relatively) sane.

A special thank you (yet again) goes to Eloise. Not only were you an amazing R.A. to have in the lab (and you taught me so many things that I don't even think you are aware of), but you were most importantly an amazing friend and supporter. I don't think I could have asked for a better PCR buddy, moral support when running gels, or company for the long hours in the lab. For all your help and kindness I am forever grateful.

To my fellow PhD students, thank you for your constant support and friendship over the years. You have become like another family and I don't know how I would have gotten through this without you. I would especially like to thank Sarah, Emily, and Jess who have been through this with me from the start and have been always been there with their support. Special shout-out again to Sarah for the amazing dance parties in the lab, Sconedog for the chats and lunch-dates, and Jess for being my PCR buddy. Massive thanks also go to my 'Pleb' Lewey who has always been there and given 200% of his time if I needed him. Thanks for keeping me both amused, supported, and (somewhat) sane (OOOoooooOOO!).

Thanks also goes to Santon for the chats, friendship, and especially the memes; and special mention goes to Adrian who became my out-of-hours (and sometimes beer and ice-cream) buddy – thanks for making the early mornings, late nights, and weekends that little bit less lonely.

I am also especially grateful to Nic who has been an amazing source of love and support when I have needed it the most. Thank you for always making time for me, encouraging me, and supporting me no matter what and even from far away, both throughout the writing of this thesis (maybe now I will finally let you read it) and in my life outside of it.

Lastly I would like to give a massive thanks to my family, who without them I don't think this would have been possible. The largest thanks goes to you. Thank you for your never-ending love and support throughout this whole time and always being there for me and willing to go above and beyond whenever I needed you. I will always be extremely grateful for everything you have done for me.

STATEMENT

The work in the present thesis has exclusively been for the use of a Ph.D. in the area of biomedical research. The data in this thesis has not been used for any other higher degree or graduate diploma in any other university. All experimental and written work is my own, except which has been referenced accordingly and all experimental work abides by the Australian ethical conduct codes regarding animal experimentation.

AASCHA BROWN

AUTHORITY OF ACCESS

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ABSTRACT

Obesity-related insulin resistance is accompanied by impaired microvascular recruitment within skeletal muscle. Loss of recruitment within the microvasculature of the muscle has been shown to be an early defect leading to impaired insulin sensitivity and in time skeletal muscle insulin resistance, although the mechanism of impairment is unknown. Both obesity and insulin resistance have been associated with chronic low-grade inflammation. A number of inflammatory factors have been shown to directly influence vascular and myocyte responses to insulin and may therefore be a contributing factor towards the development of insulin resistance. The aim of this thesis was to investigate whether impaired vascular and metabolic responses to insulin are attributed to the presence of inflammation.

Dietary models of obesity-induced insulin resistance were studied in Sprague Dawley rats. Rats were placed on diets of different fat content (41% and 58% calories derived from fat) in addition to a cafeteria-style diet. All diets were given for a 4 week duration, with both high fat (58%) and cafeteria-style diets also extended to 12 weeks. Hyperinsulinemic euglycaemic clamps and 1-methyl xanthine (1-MX) techniques were used to measure whole body insulin sensitivity and microvascular recruitment, with inflammatory gene expression in the skeletal muscle and epididymal adipose tissue measured by quantitative real-time PCR (q-PCR).

Four weeks of 41% high fat diet caused obesity, insulin resistance, and impaired capillary recruitment in rats; however inflammatory markers in epididymal fat were not altered. Raising dietary fat content to 58% resulted in increased adiposity of the epididymal fat pad but did not cause insulin resistance or inflammation of adipose tissue. Long term feeding with this diet attenuated obesity and these animals did not differ from paired control diet rats in insulin sensitivity or adipose tissue inflammation. A more palatable and varied cafeteria diet resulted in even greater obesity and insulin resistance than the 41% high fat diet and this was sustained after 12 weeks of feeding. However this diet did not cause inflammation after either 4 or 12 weeks of dietary intervention. Finally, restoration of muscle insulin sensitivity by metformin in 41% high fat fed rats was tested to see if adipose tissue inflammation could be reduced. Metformin was found to significantly improve insulin sensitivity in insulin resistant rats after 4 weeks of high fat feeding with the 41% diet, and significantly improve skeletal muscle microvascular recruitment compared to that of control animals. Therefore in addition to its known glucoregulatory actions, this study has shown metformin to have

significant actions directly on the vasculature and can restore microvascular blood flow within insulin resistant skeletal muscle. However, these improvements were not accompanied by attenuation of adipose inflammatory gene expression.

The lack of up-regulated inflammatory responses in insulin-sensitive tissues of obese and insulin resistant rats suggests that inflammation may not be a driving factor for the development of metabolic or vascular dysfunction present in insulin resistance in rats. The lack of inflammatory response despite significant obesity suggests that rats may possess mechanisms protecting against obesity-induced inflammation. Adipose tissue microenvironment and expansion have been identified as potential mechanisms regulating the induction of inflammation with evidence suggesting that adipose tissue can undergo both healthy and unhealthy expansion in response to lipid accumulation. Data presented in this thesis suggest that in rats adipose tissue expansion occurs in a manner that protects against the development of inflammation, although further investigation is required to identify such potentially protective mechanisms involved in obesity-induced adipose tissue expansion.

ABBREVIATIONS

1-MX	1-Methylxanthine
2-DG	2-deoxy-D-[1-^{14}C] glucose
ANOVA	Analysis Of Variance
CAF	Cafeteria Diet
ET-1	Endothelin-1
eNOS	Endothelial Nitric Oxide Synthase
EMR1	EGF-like Module-containing Mucin-like Hormine Receptor-like 1
FFA	Free Fatty Acid
GIR	Glucose Infusion Rate
GLUT-4	Glucose Transporter 4
HFD	High Fat Diet
iNOS	Inducible Nitric Oxide Synthase
INS	Insulin
IRS	Insulin Receptor Substrate
L-NAME	N(G)-Nitro-L-Arginine Methyl Ester
MET	Metformin
MCP-1	Monocyte Chemoattractant Protein-1
NO	Nitric Oxide
PCR	Polymerase Chain Reaction
qPCR	Real Time Polymerase Chain Reaction
SEM	Standard Error of the Mean
TNFα	Tumour Necrosis Factor Alpha

PREFACE

Some of the data obtained in the present thesis has been presented at scientific meetings and are listed below.

Posters at conferences

Australian Physiological Society Annual Meeting/Conference. Hobart, TAS. December 2015. **Aascha Brown**, Michelle A. Keske, Stephen Rattigan, Stephen M. Richards. Inflammation is not a driving factor in the development of insulin resistance in diet induced obesity in rats.

Oral presentations at conferences

Australian Diabetes Society/Australian Diabetes Educators Association Annual Scientific Meeting. Adelaide, SA. August 2015. **Aascha Brown**, Michelle A. Keske, Stephen Rattigan, Stephen M. Richards. Obesity, insulin resistance and the role of inflammation in cafeteria diet-fed rats.

CHAPTER 1

INTRODUCTION

1.1 Diabetes

Type 2 Diabetes is rapidly reaching epidemic proportions and becoming a serious health problem worldwide due to the high prevalence of obesity [1, 2]. In 2013, diabetes was predicted to affect approximately 382 million people worldwide, with this number expected to increase to 592 million by 2035. It is believed the more than 85-90% of those affected are suffering from Type 2 Diabetes [3, 4]. A study conducted by Shaw *et al* [5] estimating the global prevalence of diabetes between 2010 and 2030 predicts a 20% increase in diabetes diagnosis in developed countries within this timeframe, however most alarming is the predicted 69% increase of diabetes diagnosis to occur within developing countries [5]. Despite this prediction the increasing burden of diabetes is expected to remain within developed countries [6]. There has also been a growing trend in the prevalence of Type 2 Diabetes reported in children and adolescents [7, 8]. Twenty years ago approximately only 3% of diabetes diagnosis in children was classified as Type 2, while today that number has increased to more than 75% of childhood diabetes diagnosed as Type 2 Diabetes [7, 9, 10]. All studies investigating the development of diabetes agree it is a growing problem and considerable additional work will be placed on the healthcare system if these trends continue as Type 2 Diabetes is currently one of the leading causes of mortality in the Western world [11, 12].

Both obesity and Type 2 Diabetes are associated with the development of insulin resistance, which is characterised by impaired insulin signalling and response within insulin-sensitive tissues such as skeletal muscle [13-16]. This leads to poor uptake of glucose by the body tissues, resulting in high blood glucose levels and hyperglycaemia [13, 17]. Abdominal obesity in particular is associated with insulin resistance in skeletal muscle, adipose tissue and the liver [18, 19], with insulin resistance within these tissues often present years before the development and diagnosis of Type 2 Diabetes [20]. In addition to its metabolic actions, insulin also has important action on vascular responses by controlling blood flow and the delivery of both glucose and insulin to the skeletal muscle. In insulin resistant conditions these vascular actions are impaired which further contributes towards the development of muscle insulin resistance [15, 21-23]. This vascular dysfunction has been associated with other health complications such as cardiovascular disease, hypertension and atherosclerosis [14]. Dysfunction within large blood vessels (macrovascular) is found to occur in conditions

such as arterial disease and stroke, whilst dysfunction of smaller blood vessels (microvascular) can result in nerve damage (neuropathy), kidney disease (nephropathy) and complications and damage to the eyes (retinopathy) depending on the specific location [24].

Obesity, Type 2 Diabetes and insulin resistance are proposed as being states of chronic low grade inflammation. This presence of background inflammation is thought to be an influential factor contributing towards altered insulin signalling seen in obesity and insulin resistance [19, 25-28]. Although a number of potential mechanisms have been proposed and identified, it is unclear how rapidly inflammation develops in diet-induced obesity, and whether it has the potential to impair microvascular responses at early stages of insulin resistance and thus contribute towards impaired metabolic responses. A number of studies propose inflammation to be a potential driving force which initiates the dysfunction present in these states [18, 27-29]. Significant progress has been made to understand how and why insulin resistance and the subsequent progression to diabetes develops [15, 18, 22, 30-32], however the exact mechanisms by which insulin resistance occurs at a molecular and signalling level, in both the skeletal muscle and vasculature, still remains to be confirmed.

1.2 Insulin

Insulin is a hormone produced by the pancreas in response to high levels of glucose in the blood [33, 34], such as after a meal. In order to maintain glucose homeostasis, insulin promotes skeletal muscle and adipose tissue to take up glucose from the blood, while suppressing hepatic glucose output by the liver [33]. Skeletal muscle is of high importance for returning blood glucose levels to fasting levels as it accounts for approximately 80% of whole body insulin-mediated glucose uptake following a meal [35]. In liver and skeletal muscle, glucose is stored as glycogen – a long-term energy store that can act as a reservoir of glucose for muscle cells when availability is low [36, 37]. Liver glycogen is able to provide glucose to the rest of the body for fuel when circulating blood glucose levels fall via gluconeogenesis [38].

The actions of insulin depend on its binding to specific receptors which are expressed on the surface of most cells in the body [39, 40]. This binding initiates a complex signalling cascade collectively known as the insulin signalling pathway [41] (Figure 1.1). Insulin is able to signal through a number of pathways including Grb/Sos, MAPK, and TOR, however the most relevant signalling pathway for metabolism is by PI3K/Akt. The insulin signalling pathway is highly complex and consists of multiple feedback loops and interactions between signalling branches and receptors [42, 43]. Many previous studies have investigated this response in order to gain understanding of the signalling pathways by which insulin promotes glucose uptake and allow greater insight into insulin's actions within various tissues. [22, 34, 39-41, 44-49].

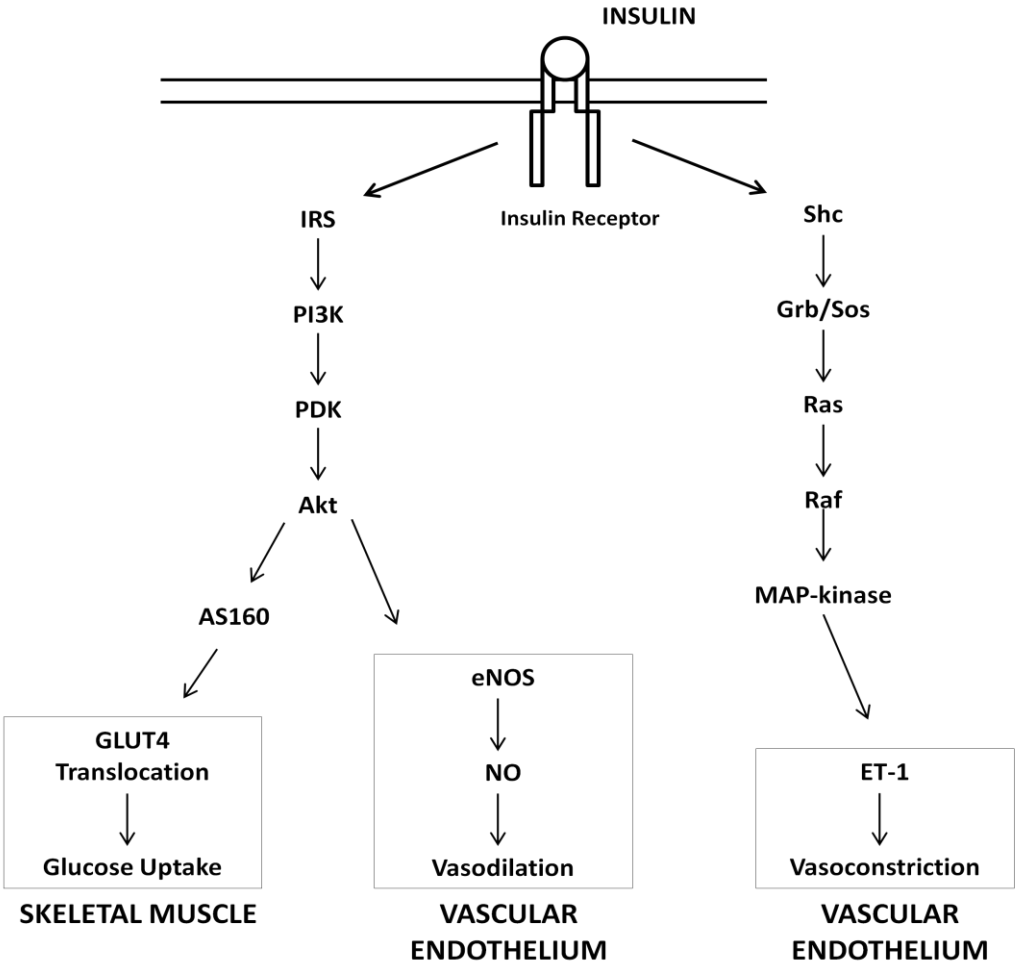

Figure 1.1 – Comparison of metabolic and vascular insulin signalling pathways

1.2.1 Metabolic actions of Insulin

The insulin signalling pathway is initiated by the binding of insulin to the insulin receptor (IR) which activates tyrosine domains and causes its auto-phosphorylation [45]. The IR becomes activated and is able to bind and phosphorylate insulin receptor substrate proteins (IRS) which act as docking sites for other signalling molecules downstream in the pathway [45, 49]. Tyrosine phosphorylation of IRS creates recognition sites to allow binding of phosphatidylinositol 3-kinase (PI3K) at SH2 domains [50], resulting in the activation of PI3K and generation of phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 activates a series of serine kinases including 3-phosphoinositide-dependent protein kinase-1 (PDK-1) which becomes activated and can then go on to phosphorylate and activate other serine-threonine kinases downstream in the pathway such as Akt [2, 40, 51-55]. Activated Akt is then able to phosphorylate AS160. Phosphorylation of AS160 is required for GLUT4 translocation to the cell surface, allowing for glucose uptake in the skeletal muscle and adipose tissue [56-60] (Figure 1.1). This translocation of GLUT4 is a major rate-limiting step for glucose uptake in skeletal muscle and adipose tissue [56, 61, 62]. Inhibition or altered associations of any of the key signalling molecules in the PI3K signalling pathway have the potential to prevent GLUT4 translocation and therefore limit the amount of glucose that can be taken up by the cells and tissues in response to insulin. Akt is also able to activate glycogen synthesis by inhibiting GSK-3β so that glucose it taken up by adipose and skeletal muscle, and can be stored for future energy use when circulating glucose levels fall [52, 63, 64].

As outlined, insulin not only stimulates the PI3K signalling pathway to initiate glucose uptake and removal from the circulation, but it also has actions on the vasculature to promote vasodilation [47, 51, 65]. In the vascular endothelium, insulin is able to signal through the PI3K pathway to stimulate the production of the potent vasodilator nitric oxide (NO) [47, 51, 66]. This resulting vasodilation increases blood flow to skeletal muscle thereby allowing for enhanced glucose uptake [23, 67]. As well as being a potent vasodilator, NO also functions as an important signalling molecule in other pathways and can be produced by almost all cells in the body [68-70]. There are three nitric oxide synthase (NOS) isoforms: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). Both nNOS and eNOS isoforms are

expressed constitutively, while as its name suggests, iNOS expression occurs only when it is induced, usually by inflammation [68, 70-72].

Insulin-initiated production of NO occurs through activation of the PI3K signalling pathway and shares similar initial signalling cascades as those required for glucose uptake by skeletal muscle – the binding of insulin to the IR, phosphorylation of IRS followed by binding and phosphorylation of PI3K. PI3K stimulates the production of PIP_3 which phosphorylates and activates PDK which in turn phosphorylates and activates Akt [13, 73]. However, the difference in the signalling pathway between the metabolic and vascular actions of insulin differs at this key signalling molecule as instead of Akt activating GLUT4 resulting in the subsequent uptake of glucose by myocytes, Akt activates another branch of signalling by directly phosphorylating eNOS on the serine 1177 residue [51, 74, 75] which stimulates eNOS to release NO, resulting in vasodilation (Figure 1.1). This is an important action of insulin as increased vasodilation results in increased blood flow to skeletal muscle, which not only increases the delivery and uptake of glucose by skeletal muscle but also increases the delivery of insulin itself to facilitate glucose uptake and insulin delivery to myocytes [44, 51].

Insulin is able to signal through a pathway other than PI3K to elicit other vascular responses such as vasoconstriction [2]. Insulin is able to signal through the MAP-kinase (MAPK) pathway (Figure 1.1) to activate the secretion of endothelin-1 (ET-1), a powerful vasoconstrictor, in the vasculature [66]. This signalling acts concurrently with insulin's ability to promote vasodilation through eNOS in order to maintain vascular function and provide regulation of blood flow by controlling the dilation and constriction of the vasculature. Following the activation and auto-phosphorylation of the insulin receptor (IR), insulin is able to phosphorylate and bind to insulin receptor substrates (IRS). Tyrosine phosphorylated IRS binds to SH2 domains and activates Sos, which in turn activates the GTP binding protein Ras and begins a series of kinase phosphorylation cascades involving Raf and MAPK [41, 55, 76, 77]. It is this activation which stimulates ET-1 to be produced. However, signalling through the MAP-kinase pathway to produce ET-1 has not been as extensively studied as insulin signalling through the PI3K pathway to produce NO, so little is known about the exact details of how ET-1 secretion is regulated [72]. Despite this, these mechanisms of insulin signalling in the vasculature are highly important in regulating the net

vascular response to insulin [66, 78]. An imbalance of vasodilation or vasoconstriction in the vasculature may lead to impaired vascular delivery and as a result, the development of insulin resistance [2, 31, 78-81].

1.2.2 Vascular actions of Insulin

Insulin is reported to have two types of control within the vasculature of skeletal muscle: to increase artery blood flow and to increase microvascular capillary recruitment [22, 82, 83]. In the past, increased total blood flow has been suggested to be important in insulin-mediated glucose uptake in skeletal muscle since it was thought to enhance the delivery of insulin and glucose to the muscle cells [84]. However it has now come to light that the distribution of blood flow within skeletal muscle may have a more significant effect on glucose uptake than bulk flow itself [16, 21-23, 32, 44, 56, 85, 86]. These studies suggest that microvascular blood flow is critical for glucose uptake within the skeletal muscle as opposed to increased total blood flow. Insulin-mediated vasodilation occurs in distinct stages. Terminal arterioles are dilated first, resulting in an increased number of capillaries receiving blood flow. This process is referred to as microvascular recruitment and leads to a redistribution of blood flow in muscle without altering total blood flow to the muscle. Following this, relaxation of larger vessels results in an increase in the overall net blood flow to muscle [22, 23, 87]. This microvascular action of insulin was first characterised in the skeletal muscle of rodents [23] and again more recently confirmed in the human forearm [85, 88].

A concept championed by Clark and Rattigan *et al* [23] is that capillary recruitment occurs via redistribution of microvascular blood flow between two flow routes. Early studies using the rat perfused hindlimb support the idea of two flow routes being present in skeletal muscle, nutritive and non-nutritive flow [67, 83, 89]. One flow route has close contact with the myocytes and is able to exchange nutrients easily so is regarded as the nutritive flow path. The other flow route has very little to no contact with myocytes and instead has more interaction with connective tissue and adipocytes. Because of this, it is viewed as non-nutritive flow. It has been shown that non-nutritive vessels have the ability to carry a flow reserve which allows blood to be redistributed into the nutritive flow paths during activities considered to be of high metabolic demand, such as during exercise [67, 89]. This

redistribution of flow into a nutritive flow path where there is more contact with myocytes allows for a greater uptake of glucose from the blood into muscle cells. Because of this, insulin has been shown to have the capacity to change and redistribute blood flow from the non-nutritive to a nutritive flow route so that there is greater contact and opportunity for glucose to be taken up by the skeletal muscle [22, 67, 89].

In healthy conditions, insulin is believed to increase total blood flow and microvascular recruitment via a PI3K-dependent pathway in order to enhance glucose uptake by muscle cells [44]. However, it is difficult to confirm the exact processes that lead to microvascular recruitment occurring because a functioning intact model with signalling between skeletal muscle and microvasculature is needed. Therefore insulin-mediated microvascular recruitment has largely been studied *in vivo* [22, 23, 32, 90, 91]. Such studies have shown that insulin has the ability to increase total blood flow and microvascular recruitment in skeletal muscle, but it is this microvascular recruitment that has been revealed to be responsible for increasing muscle glucose uptake [23, 32]. Inhibition of PI3K with wortmannin has been found to block insulin-mediated microvascular recruitment [90]. Insulin-mediated glucose uptake is also found to be inhibited when microvascular recruitment is blocked by α-methyl serotonin or by the nitric oxide synthase inhibitor N(G)-Nitro-L-arginine methyl ester (L-NAME) [92-94]. Vasodilators have been shown to increase blood flow in both normal and insulin resistant skeletal muscle, however this increase in flow was not shown to enhance glucose uptake [16, 23, 92]. A study by Mahajan *et al.* [95] comparing nitric oxide vasodilators showed that the vasodilators bradykinin and methachonine both increased blood flow, however only methacholine enhanced microvascular recruitment and skeletal muscle glucose uptake. These results indicate that methacholine acts upon different specific sites within the muscle vasculature in order to enhance recruitment as opposed to other vasodilators which do not. Currently methacholine is the only vasodilator that increases both total blood flow and insulin-mediated glucose uptake in the skeletal muscle [16, 78]. From these data, recruitment of the vasculature appears to be independent of changes in total blood flow which are also caused by insulin [44, 82, 87]. [82, 87]. It has even been observed that insulin-mediated microvascular recruitment occurs before changes in total blood flow in muscle [32]. This raises the possibility that insulin may have the potential to use different mechanisms to recruit macrovessels which control total blood flow and microvessels which control capillary flow in the muscle [87]. Further studies into microvascular flow and

insulin-mediated vascular recruitment are needed to bring more clarity to this area of research.

1.3 Insulin Resistance

Insulin resistance is characterised by decreased sensitivity of tissues to insulin. Insulin is therefore unable to suppress hepatic glucose output by the liver or initiate glucose uptake in skeletal muscle and adipose tissue [13, 37, 48, 52, 96]. This leads to high circulating levels of glucose in the blood, known as hyperglycaemia, which has been shown to be involved in the signalling dysfunction and chronic inflammation present in insulin resistant states [2, 13, 97]. This dysfunction in insulin response may be present for a number of years before the development of abnormal plasma glucose levels which lead to the diagnosis of insulin resistance and Type 2 Diabetes, as well as other conditions such as hypertension and cardiovascular disease [91, 98].

1.3.1 Metabolic Insulin Resistance

During the development of insulin resistance it has been proposed that the liver and skeletal muscle both become resistant to the actions of insulin at an early stage. As insulin resistance progresses, insulin is unable to suppress glucose output by the liver or stimulate glucose uptake by the skeletal muscle. This results in the glucose concentrations within the blood to rise, which in turn stimulates the pancreas to increase insulin secretion in order to try and maintain euglycaemia. This is often referred to as compensatory hyperinsulinemia. Initially this increased insulin secretion by the pancreas is able to maintain normal blood glucose concentrations, however over time either the production of insulin by the pancreas or the responsiveness to insulin action itself is reduced, resulting in hyperglycaemia that can no longer be controlled by normal means.

Insulin resistance is characterised by the impaired insulin response or insulin signalling. It has been identified that skeletal muscle from obese and insulin resistant subjects show selective impairment in insulin signalling pathways; specifically through the PI3K/Akt

pathway as opposed to the MAPK pathway which maintains normal insulin signalling responses. Selective defects within the PI3K signalling pathway specifically inhibit the stimulation of glucose uptake by skeletal muscle and adipose tissue, in addition to insulin-mediated vascular responses [30, 99, 100]. Impairment of insulin signalling within this pathway may be due to a defect of the insulin receptor itself or in receptor expression, ligand binding, phosphorylation and tyrosine kinase activity of other downstream signalling molecules such as IRS, PI3K, Akt or GLUT4 [33, 101]. Reduced signalling through the PI3K pathway results in reduced activation of GLUT4, which is a major rate-limiting step for glucose uptake in skeletal muscle and adipose tissue as reduced GLUT4 translocation limits the amount of glucose that can be taken up by the cell and tissue [56]. In regards to implications on vascular responses, impaired insulin signalling through the PI3K pathways also limits the production of nitric oxide in order to stimulate vasodilation within the vasculature. As insulin signalling through the MAPK pathway remains unaffected this means that the production of ET-1, and thus vasoconstriction, is unaltered which can results in the dysfunction of vascular responses within the endothelium often characterised in obese and insulin resistant subjects.

Insulin resistance has also been characterised by the increased presence of increased fatty acids and pro-inflammatory factors such as cytokines and macrophages. Obesity and insulin resistance are associated with increased lipolysis within the adipose tissue, resulting in an increased production of fatty acids. It has been proposed that increased circulating levels of free fatty acids (FFA) may contribute towards the development of insulin resistance [91, 102-104]. In healthy states, insulin stimulated the synthesis and storage of fatty acids in adipose tissue, but in insulin resistant conditions, the adipose tissue becomes insulin resistant itself, so the uptake of FFA becomes impaired, resulting in the rise of circulating FFA levels in the plasma [105]. Consequently FFA and triglycerides have been shown to accumulate in non-adipose tissues, such as skeletal muscle, due to their high levels in the blood. This uptake of FFA is thought to occur due to altered function of lipid transporter proteins, suggesting an impairment in fat disposal as much as an increased uptake of FFA and triglycerides by other tissues [106]. This accumulation of FFA within insulin-sensitive tissues is closely associated with insulin resistance. This type of exposure of skeletal muscle to high cellular levels of

FFA can result in impaired insulin signalling by preventing full activation of signalling molecules in the PI3K pathway, such as IRS, PI3K, PDK or Akt [103, 107].

Exposure to FFA and triglycerides increases levels of diacylglycerols (DAGs) and ceramides, which can activate kinases such as PKC, IKKβ, and JNK, as well as transcription factors such as NF-$_K$β. Each of these in turn have been found to cause insulin resistance by inhibiting insulin signalling at various sites [18, 29, 108, 109]. Increased expression of JNK and IKKβ have been found in the liver [18, 110], as well as skeletal muscle and adipose tissue during obesity [18, 111]. Evidence to support their activation in the development of insulin resistance can be given through the use of transgenic animal models. Transgenic knockout of JNK results in the improvement of insulin sensitivity of high fat fed mice and the development of obesity [111], whilst transgenic mice with constitutively activated IKKβ transgene within the liver have impaired hepatic insulin sensitivity, impaired skeletal muscle insulin sensitivity, hyperglycaemia, and increased liver inflammation [112].

Exogenous infusions of FFA and DAG have been shown to block the activation of IRS in skeletal muscle of rodent models [103, 107, 113]. The modifications to insulin signalling include reduced tyrosine phosphorylation of IRS and increased IRS serine phosphorylation. This decreases the ability of IRS to associate with PI3K, thereby reducing its activity and resulting in a decreased ability of insulin to stimulate glucose uptake in skeletal muscle. Muscle samples taken from Type 2 Diabetic and insulin-resistant obese subjects have been shown to have this decrease in IRS tyrosine phosphorylation and reduced IRS/PI3K association in response to insulin [114, 115]. As well as their association with the development of muscle insulin resistance, increased levels of FFA, DAG, ceramide and kinases are also associated with endothelial dysfunction and inflammation [18, 19, 29, 91, 116-119]. Insulin resistance has also been associated with the accumulation of fat within other tissues such as the liver and skeletal muscle. This increased fat in non-adipose tissue can result in lipotoxicity which can further contribute towards the dysfunction of both metabolic and vascular actions.

Insulin resistance has also been strongly associated with the increased production and initiation of inflammatory factors within the adipose tissue [26, 27, 120-124]. Their increased expression has been proposed to contribute towards impairing both insulin-mediated metabolic and vascular responses. A number of inflammatory factors including iNOS, TNFα, IL-6, and macrophage chemotactic factor MCP-1 have been shown to impair the normal actions of insulin within both the muscle and vasculature [26, 29, 125-130]. Exogenously infused TNFα has also been shown to impair vascular responses such as capillary recruitment in non-obese rats [126]. This suggests that the vasculature has the potential to be highly sensitive to inflammatory responses [22, 23, 131-133]. Although obesity-associated inflammation has been investigated within the literature, much is still unknown about the mechanisms surrounding its induction in insulin resistant states. So far the contribution of inflammatory factors towards the development of vascular insulin resistance has only been partially investigated, and it is not clear whether microvascular blood flow responses within skeletal muscle are affected by this increased inflammation expression. A time course examining the relationship between inflammation and impaired insulin sensitivity, especially within the vasculature, as a result of diet-induced obesity has not been researched by others studying blood flow and microvascular recruitment as of yet. Due to shared insulin-mediated signalling pathways, it is logical that inflammation can have a significant effect on vascular responses within muscle.

1.3.2 Vascular Insulin Resistance

Obese or Type 2 Diabetic subjects are regularly found to have some form of irregularity in endothelial or vascular function [16, 91, 134]. Diabetic individuals have shown to have reduced production and response to NO even before diagnosis, and it is extremely likely that some impairment to endothelial function has already been established early in the disease progression [91]. Endothelial dysfunction is defined as the partial or complete loss of balance between vasoconstriction and vasodilation of blood vessels [91, 135, 136]. It is considered to be an important event in the development of vascular complications present in diabetes and the development of insulin resistance. By the time Type 2 Diabetes is diagnosed, it is expected that endothelial function is already severely impaired [91].

Insulin resistance not only results in impaired metabolic actions to stimulate glucose uptake, but also a reduced ability of insulin to signal through the PI3K pathway to initiate NO production and vasodilation [13-16, 44, 56, 116, 137]. Although insulin has impaired signalling through the PI3K pathway, the other branch of insulin signalling in the vasculature via MAPK remains unaffected or even enhanced in insulin resistance [2]. Therefore, insulin is able to signal through the MAPK pathway, resulting in increased ET-1 secretion and heightened vasoconstriction of the vasculature. The imbalance between signalling through the PI3K and MAPK pathways contributes to both the impaired vascular and metabolic actions of insulin [81]. Insulin's ability to increase blood flow and glucose uptake in skeletal muscle has been found to be inhibited in insulin resistant, obese and diabetic subjects due to the imbalance and altered vascular responses that occur as a result of the inhibition of insulin signalling through the PI3K pathway [117].

It has been proposed that the insulin resistance in skeletal muscle is at least partly due to impairment of insulin-mediated vascular responses [56, 136, 138]. This is because before insulin can stimulate the PI3K signalling pathway to initiate glucose uptake in muscle cells, insulin first needs to be delivered to the muscle via the vasculature [49, 56]. An increase in blood flow and vascular recruitment in skeletal muscle is dependent on the production of NO in the vasculature for vasodilation [32, 139, 140]. In insulin resistant conditions, there is a defect in insulin's ability to signal through the PI3K pathway, which prevents the production of NO in the vascular endothelium. This results in decreased vasodilation and blood flow which in turn impairs the delivery of insulin and glucose to the skeletal muscle. Therefore insulin resistance can be characterised by impairment of PI3K signalling in both metabolic and vascular insulin target tissues [139, 141]. Expression of pro-inflammatory cytokines such as TNF-α and accumulation of FFA have been found to alter insulin vasodilator signalling by reducing the binding and activation of PI3K in response to insulin, resulting in reduced activation of downstream effectors such as Akt and eNOS in endothelial cells which normally activate the production of NO [18, 28, 91, 117].

1.3.3 Inflammation in Insulin Resistance

Obesity associated with Type 2 Diabetes has been shown to coexist with a state of chronic low grade inflammation, and insulin resistance related to obesity has been found to correlate with this chronic tissue inflammation [19, 26, 119, 124]. Studies that have specifically altered the degree of inflammation have noted reciprocal effects on insulin sensitivity. Many studies investigating genetic knockout models for inflammatory markers such as iNOS and MCP-1 have shown a significant improvement in whole body insulin sensitivity in response to high fat feeding compared to wild-type controls [71, 122, 127, 129, 142]. In comparison, studies have also shown that acute infusion of markers such as TNFα and IL-6 can directly impair insulin-mediated glucose uptake by the skeletal muscle as well as insulin-mediated vascular responses in non-obese animals [126, 130, 143, 144]. It is therefore assumed that this inflammatory state contributes to insulin resistance, however there is some debate as to whether inflammation aids in the development of insulin resistance, or if insulin resistance itself initiates inflammation in diet-induced obesity [26]. Numerous studies have attempted to determine why inflammation occurs in insulin resistant states related to obesity, and what implication this has on its development and progression. Rodent models fed a high-fat diet to induce obesity have been used to assess the natural development of inflammation and impaired insulin response in vascular tissue, liver, adipose tissue and skeletal muscle [26, 117].

Obesity has been associated with high infiltration and accumulation of macrophages within the adipose tissue. Macrophages have the ability to induce and secrete a range of pro-inflammatory cytokines which cause tissue inflammation and signal the production of other pro-inflammatory molecules [19, 26, 27, 119, 124, 145]. Therefore the accumulation of macrophages into adipose tissue is a potential sign that an inflammatory response has already been initiated. Monocyte chemoattractant protein 1 (MCP-1) is an adipokine that recruits the infiltration of macrophages and other pro-inflammatory cells into tissues to elicit an inflammatory response. It has been found to have significant expression within adipose tissue in obese states [122]. Studies have shown that skeletal muscle cultured with MCP-1 results in reduced glucose uptake by the muscle cells [146] and that MCP-1 knockout mice fed a high fat diet show reduced accumulation of macrophage markers within the adipose tissue, and whole body insulin sensitivity of these mice are improved compared to high fat

fed controls [122]. These results suggest that MCP-1 is influential in the induction of inflammation in obesity due to its ability to recruit macrophages to induce a pro-inflammatory response, however MCP-1 may also play a direct role towards impairing insulin-mediated responses. Some of the inflammatory cytokines induced by macrophages and reported to have strong associations with obesity and insulin resistance include TNF-α, IL-1β and IL-6 [18, 124, 126, 130].

Cytokines IL-6 and IL-1β are strong pro-inflammatory signals which can be used as markers and predictors of Type 2 Diabetes in humans [147]. Both have been widely studied and are closely linked to inflammation and the development of endothelial dysfunction [27, 124, 145, 148]. Visceral fat accumulation, which is highly correlated with obese states and insulin resistance, has been shown to be an important site for the secretion of IL-6, with its production being at least three times higher in abdominal adipose tissue that in subcutaneous tissue [118]. Exogenous infusion of IL-6 *in vivo* has shown to reduce insulin-mediated glucose uptake in the skeletal muscle by reducing association of IRS with PI3K by promoting serine phosphorylation instead of tyrosine phosphorylation on the IRS. IL-6 has also been reported to inhibit gene transcription of IRS, GLUT4 activation, as well as increase the level of fatty acyl-CoA in the skeletal muscle [130, 144].

TNF-α is a pro-inflammatory cytokine which is produced by macrophages, but is also found to be highly expressed in adipose and skeletal muscle of obese and insulin resistant subjects [18, 102, 149], and is believed to contribute towards these conditions and the development of Type 2 Diabetes [124]. Treatment with TNF-α in the skeletal muscle and liver results in decreased tyrosine phosphorylation of IRS and increased serine phosphorylation instead. Phosphorylation of serine residues on IRS interferes with insulin-stimulated tyrosine phosphorylation by the IR [150, 151]. This type of modification to IRS prevents further associations with downstream effectors in the insulin signalling pathway, such as PI3K, in response to insulin [152]. TNF-α and FFA have been shown to reduce insulin's ability to recruitment the microvasculature in the skeletal muscle [13]. Increased levels of TNF-α has been found to downregulate vasodilator pathways by causing reduced binding and activation of PI3K in response to insulin [152], resulting in reduced activation of downstream effectors such as Akt and eNOS in endothelial cells which activate the production of NO [2]. Acute

infusion of TNF-α [143, 152] and FFA [103, 107] into rodent models leads to insulin resistance, decreased glucose uptake and reduced microvasculature recruitment. It has been found that mice lacking TNF-α or TNF-α receptors have better insulin sensitivity if fed a high fat diet or are genetically altered obese models [149]. Acute infusion of TNFα has been shown to directly impair insulin-mediated haemodynamic responses in non-obese rats by preventing capillary recruitment, thereby resulting in decreased whole body insulin sensitivity due to the direct impairment of glucose uptake by skeletal muscle [126, 143]. This demonstration of exogenous TNFα being able to significantly impact vascular responses without obesity-associated insulin resistance highlights the sensitivity of the vasculature to inflammatory responses.

Inducible nitric oxide synthase (iNOS) is one of the 3 nitric oxide synthase (NOS) isoforms, and the only one which is Ca^{2+}-independent [153]. It is also the only NOS isoform expressed when it is specifically activated, in contrast to the other isoforms which are constitutively expressed [56, 72]. It was first identified in macrophages and was found to contribute to the cytotoxic actions they have in immune cells, but iNOS is now known to be expressed at low levels in other tissues of healthy individuals [154]. However high expression of iNOS appears to be detrimental with induced iNOS expression found in a number of conditions associated with inflammation such as diabetes, atherosclerosis, obesity and insulin resistance [149, 155, 156]. Increased iNOS expression is promoted by pro-inflammatory cytokines such as TNF-α and IL-6, which are also reported to be present in insulin resistant states [72, 157, 158].

High levels of iNOS expression have been observed in skeletal muscle of mice placed on high fat diets, with similar increases in iNOS expression seen in skeletal muscle of patients with Type 2 Diabetes [156, 159]. This over-expression of iNOS in genetic and high-fat diet models of obesity has been shown to play a crucial part in the development of insulin resistance. Disruption of iNOS in knockout mouse models has shown to be prevented from developing obesity-induced insulin resistance body wide [142, 149]. Although they develop obesity if placed on a high fat diet, iNOS knockout mice otherwise show protection from insulin resistance induced by high fat feeding with improved glucose tolerance and insulin sensitivity [149]. This may be due to insulin signalling through the PI3K pathway appeared

to be intact in these animals, with no alterations in phosphorylation, association or activation of any of the key signalling molecules [149]. This suggests that in normal high-fat models, iNOS impairs insulin signalling to PI3K, preventing activation of Akt which is important for glucose transport signalling, resulting in decreased glucose uptake. A similar increase in iNOS expression has also been seen in the skeletal muscle of patients with Type 2 Diabetes which may contribute to the disease state [156, 159]. iNOS is able to produce 1000-fold more NO than any other NO isoform and once it is expressed, its activity is continuous for many hours [153]. Therefore, if iNOS expression is induced in inflammatory and insulin resistant states, it is possible that the excessive NO production interferes with the highly coordinated actions of the vasculature and contributes towards the endothelial dysfunction seen in such conditions [158, 160-162]. The rise of pro-inflammatory cytokines associated with inflammation and insulin resistance may also alter the expression of iNOS in the vasculature [72], which would affect the regulation of vascular function. Increased iNOS expression is believed to interfere with the highly coordinated actions of the vasculature [158, 160-162], however its involvement in vasoconstrictor responses is less studied than the involvement it has in insulin-mediated glucose uptake in insulin resistance [142, 154].

Inflammation has not only been found to contribute to the formation of insulin resistance but also to endothelial dysfunction of the vasculature [13, 14, 16]. While there have been reports from high fat-fed mouse models that inflammation is a late development, changes in inflammatory markers have been shown to also occur early in the time course, albeit much smaller changes [26]. It is unclear from rat models and human obesity how rapidly inflammation develops, and thus whether it has the potential to impair microvascular responses at early stages of insulin resistance. In a study by Kim *et. al* [117], it was found that the vascular tissue is affected much sooner in the development of diet-induced obesity than other tissues involved in glucose metabolism. A number of inflammatory markers have been shown to have direct effects on vascular responses suggesting they can impair blood flow as soon as they appear. As discussed, both TNFα and iNOS have both been shown to alter vascular signalling [72, 126-128, 143, 160, 161]. Acute infusion of TNFα has been shown to directly impair insulin-mediated blood flow responses by preventing the recruitment of the microvasculature [126, 143], whilst excessive nitric oxide production from iNOS has been shown to interfere with the highly coordinated actions of the vasculature [72, 127, 161]. Preliminary studies conducted by Bussey (2011, University of Tasmania, PhD

thesis) also provide evidence for impaired muscle vascular responses after 4 weeks of high fat feeding that could be restored by iNOS-specific inhibition, suggesting that even at this early time point iNOS over-expression potentially impairs vascular reactivity in skeletal muscle. However despite this a direct link between the induction of microvascular impairment and the induction of diet-induced inflammation has not yet been clearly identified.

As obesity and inflammation have been closely linked within the literature, it is of no surprise that a key site of inflammatory induction is within the adipose tissue itself. This is because in addition to its role in lipid storage, the adipose tissue is highly active endocrine organ that secretes a number of hormones and adipokines [163-165]. However despite the current research within the literature, the mechanisms surrounding obesity-induced inflammation still remain elusive. It has been proposed that the adipose tissue can undergo unhealthy or unhealthy expansion in response to provide adequate nutrient storage, however the mechanisms surrounding whether healthy or unhealthy expansion occurs is still not fully understood [166]. In healthy conditions the adipose tissue undergoes hypertrophy and hyperplasia to accommodate additional lipid storage. In this state, adipose tissue expansion is associated with increased angiogenesis to supply adequate oxygen and nutrients to the expanding tissue. It is hypothesised that at times when excessive lipid storage is needed, such as during high fat feeding or obesity, adipose tissue expansion occurs at a much higher rate. This rapid expansion of the adipose tissue results in excessive adipocyte hypertrophy. It has been found that the additional remodelling that occurs during healthy expansion, such as the development of new adipocyte cells and vascular growth, either does not occur during this rapid expansion process or it occurs at a much lower rate [165, 166]. Due to their large size and inadequate oxygen availability, poorly oxygenated adipocytes can become hypoxic within the tissue. This triggers the increased expression of Hypoxic Inducible Factor 1α (HIF-1α) to the hypoxic sites [167-169]. HIF-1α has been identified as an early initiator of inflammatory responses and can signal for the induction of macrophages and the proliferation of inflammation factors in the tissue. Very poorly oxygenated adipocytes may become necrotic which also induces increased inflammatory responses and macrophages locally within the tissue [166, 170, 171]. Therefore unhealthy expansion and the development of hypoxia within the adipose tissue is a possible factor underlying the induction of obesity-induced inflammation in insulin resistant conditions. However further studies are required to confirm this proposed mechanism.

1.4 Summary of research aims

A key unanswered question regarding obesity and insulin resistance is the development of inflammation in this state and whether obesity-induced inflammation contributes towards the development of both muscle and vascular insulin resistance. A number of inflammatory molecules, such as TNFα and iNOS, have been shown to alter and impair insulin-mediated responses within both the muscle and vasculature, however currently the mechanisms regulating the induction of inflammatory responses within obesity are still not understood. The vast majority of inflammatory studies in regards to diet induced obesity have primarily been performed in mouse models, and similar studies in rats are considerably outnumbered. Therefore investigation of diet-induced obesity on inflammation and insulin resistance in rats will allow, not only for the investigation of inflammation on physiological responses such as blood flow, but also contribute to the current literature by providing further information as to its presence in rats for comparison. Therefore, this project hypothesises that inflammation is a key contributor to the development of both microvascular and skeletal muscle insulin resistance in diet-induced obesity in rats. Therefore, the aim of this thesis was to investigate the influence of obesogenic diets on the induction of adipose tissue inflammation, and the contribution of inflammation to obesity-induced muscle and vascular insulin resistance.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Male Sprague Dawley rats, approximately 4 weeks of age, were obtained from the University of Tasmania Central Animal Facility. All animals were housed in temperature and light controlled conditions (21ºC ± 1ºC) with a 12 hour light/dark cycle. Animals were provided with free access to water and commercial semi purified diets. Details of the various dietary interventions are provided in each chapter accordingly.

All experiments and procedures were approved by the University of Tasmania Animal Ethics committee and undertaken in accordance to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004).

2.2 Anaesthetised Rat Experiments

2.2.1 Surgical Procedure

Rats were anaesthetised with an intraperitoneal injection of sodium pentobarbital (50mg/kg body weight). A tracheostomy tube was surgically inserted to allow for spontaneous breathing during the experiment. Cannulas were inserted into both jugular veins to allow for intravenous infusion. Another cannula was inserted into the carotid artery and connected to a pressure transducer (Transpac IV, Abbott Critical Systems) to allow for the measurement of blood pressure. Arterial blood sampling was also performed through the carotid cannula line. The femoral blood vessels of one hindlimb were exposed and the femoral artery was carefully separated from the femoral vein and nerve. The epigastric vessel was ligated and a flow probe (0.5 mm VB series, Transonic Systems) was positioned around the femoral artery to allow for the measurement of total femoral blood flow. Both the vessels and flow probe were covered in lubricating jelly (Mohawk Medical Supply, Utica). The probe was connected to a flow meter (model T106 ultrasonic volume flow meter, Transonic Systems) which was connected to an IBM-compatible computer. Figure 2.1 outlines the surgical procedure performed.

Blood pressure, heart rate, and femoral artery blood flow were measured continuously using WINDAQ data acquisition software (DATAQ Instruments). Anaesthesia was maintained by the continuous infusion of aqueous sodium pentobarbital (0.6mg/min/kg body weight) via the

jugular vein. Body temperature of the rat was maintained at 37ºC using a heated pad and heating lamp. Completion of the surgical procedure was followed by a 60 minute equilibration period to allow for blood pressure to stabilise before a 2 hour insulin clamp was performed.

2.2.2 Muscle Glucose Uptake

Skeletal muscle glucose uptake was measured by the uptake of 2-deoxy-D-[1-^{14}C] glucose (2-DG, 0.1 mCi/mL, Perkin Elmer) as previously described [172]. In brief, 45 minutes prior to the end of the hyperinsulinemic euglycaemic clamp procedure a 200µL bolus of 2-DG was given (20µCi). Arterial plasma samples (25µL) were collected 5, 10, 15, 30, and 45 minutes after the 2-DG bolus to assess plasma clearance of 2-DG. At the end of the clamp procedure calf muscle was excised, immediately freeze clamped in liquid nitrogen, and kept at -80ºC until required. The frozen muscle was ground into a fine powder under liquid nitrogen and approximately 100mg of powdered muscle was homogenised with 1.5mL of distilled water using a Heidolph silent crusher. The homogenate was centrifuged at 13,000 rpm at 4ºC for 10 minutes and 1mL of supernatant was assessed for free and phosphorylated 2-DG using an anion exchange column (AG-1X8, Bio-Rad Laboratories). Biodegradable counting scintillant (Amersham) was combined with each radioactive sample and radioactivity was measured using a scintillation counter (Perkin Elmer). From this measurement and the disappearance of 2-DG in plasma, the rate of glucose uptake by the skeletal muscle (R'g), was calculated as described by others [172] and expressed as µg/g/min.

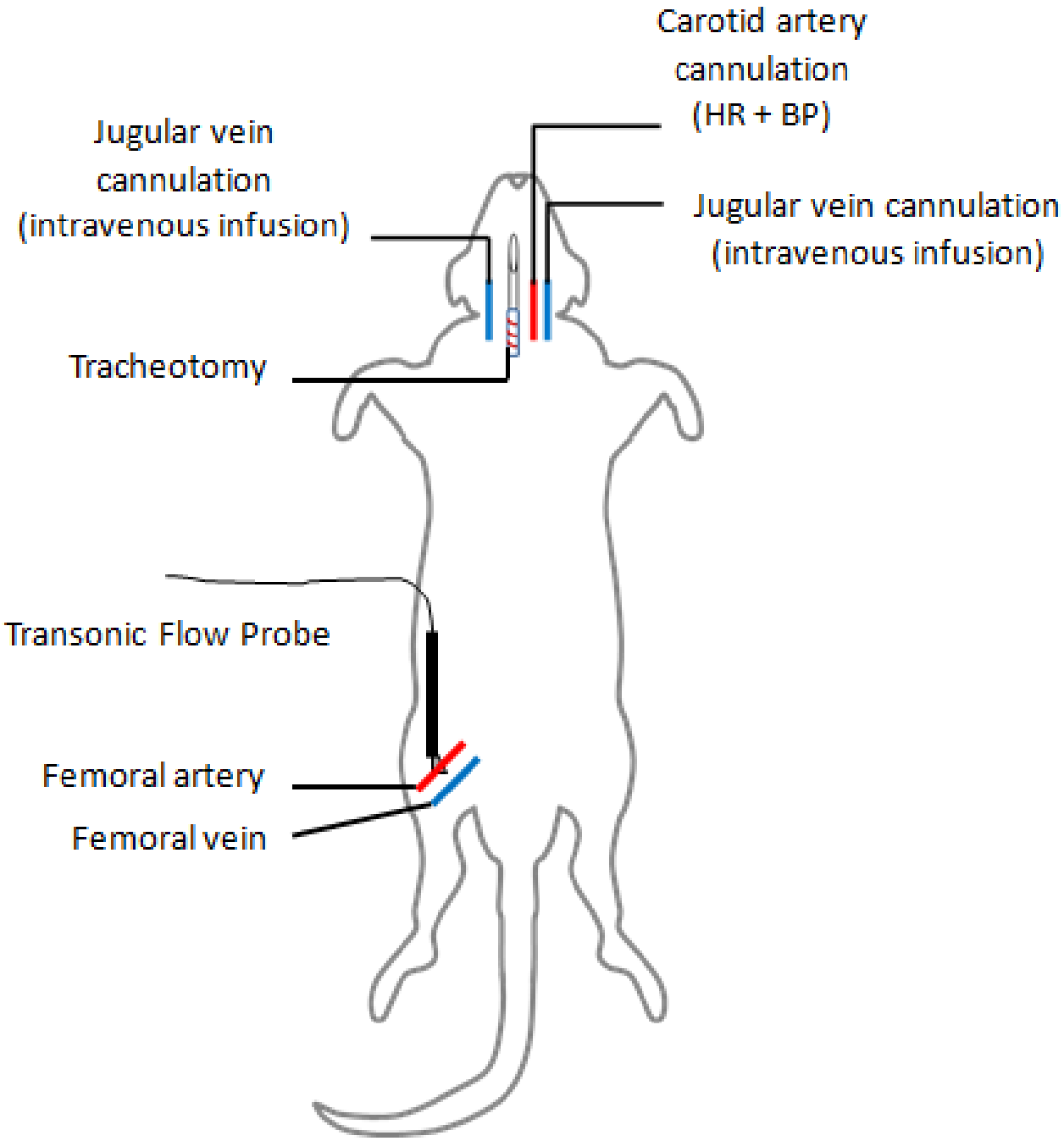

Figure 2.1 – Diagram of surgical procedure for anaesthetised rat experiments.

Following anaesthesia a tracheostomy tube was inserted and both jugular veins and the carotid artery were cannulated to allow for monitoring of blood pressure and heart rate, measurement of arterial blood glucose concentrations, and the intravenous infusion of anaesthetic, insulin, and glucose for hyperinsulinemic euglycaemic clamp experiments. The femoral artery of one hindlimb was also exposed and carefully separated and a transonic flow probe was positioned around the isolated artery to measure femoral blood flow.

2.2.3 Skeletal muscle microvascular perfusion

Microvascular perfusion within skeletal muscle was measured by the metabolism of exogenously infused 1-methyl xanthine (1-MX) as described elsewhere [23, 86]. In brief, a bolus of allopurinol (10μmol/kg, Sigma Aldrich) was given 5 minutes before 1-MX infusion in order to partially inhibit xanthine oxidase activity and ensure a constant saturating arterial level of 1-MX. Infusion of 1-MX (0.4mg/min/kg body weight) occurred for the final 60 minutes of the clamp procedure. At the end of the experiment 100μL of arterial plasma was added to 20μL of perchloric acid (2M) to precipitate the proteins. Hind-leg venous plasma was obtained from the femoral vein and 100μL was mixed with 20μL perchloric acid (2M). Samples were centrifuged for 10 minutes and the supernatant was assessed for 1-MX and oxypurinol concentration using reverse-phase high-performance liquid chromatography as previously described [23, 173]. 1-MX metabolism (nmol/min) was calculated from the difference of arteriovenous plasma 1-MX levels multiplied by femoral blood flow.

2.3 Gene Expression Experiments

2.3.1 Tissue Sample Preparations

Gastrocnemius muscle and epididymal fat pad samples were taken from anaesthetised Sprague Dawley rats at the end of hyperinsulinemic euglycaemic clamps. Tissue samples were freeze clamped in liquid nitrogen and stored at -80ºC until required. Frozen tissue samples were ground into a fine powder in liquid nitrogen and stored at -80ºC until required.

2.3.2 RNA Extraction

Total RNA was extracted from ground tissue samples using the Tri Reagent method (Sigma Aldrich). Approximately 100mg of ground skeletal muscle and 150-200mg of ground adipose tissue was used for each extraction. The RNA pellet was resuspended in 10-20μL of PCR-grade water (RNAse and DNAse free) (Bioline or Qiagen) and RNA concentration was quantified by measuring spectrophotometer absorbance at 260nm. RNA quality was also assessed and an A260/280 ratio value of ‘2’ was obtained for all RNA used in the following experiments. RNA was stored at -80ºC until required if use was not immediate.

2.3.3 Polymerase Chain Reaction (PCR)

2.3.3.1 Quantitative PCR

RNA extracted from ground tissue samples was reverse transcribed to obtain complementary DNA (cDNA) strand. Total RNA was diluted to a concentration of 1µg per reaction before being reverse transcribed and amplified per set of primers (GeneWorks Australia) using SuperScript III (Invitrogen). Reverse transcription was performed on a T100 Thermal Cycler (BioRad) and conditions consisted of 50ºC for 60 minutes, 70ºC for 15 minutes, and then sample temperature reduced to 4ºC. Samples were held at 4ºC until required.

Real time PCR (qPCR) was performed using SYBR Green (Qiagen) on cDNA obtained through reverse transcription. Incubation conditions for qPCR were are follows: 95 ºC for 15 minutes for initial denaturation followed by 35 cycles of 95 ºC for 30 seconds, 60 ºC for 60 seconds, and 72 ºC for 120 seconds followed by an extension of 81 ºC for 15 seconds to acquire fluorescence reading. All samples were run on a Rotor-gene 6000 real time PCR machine for analysis of gene expression (Corbett Research). Ct vales were obtained with a fluorescence threshold at 0.05 for all samples. A melt curve of fluorescence versus temperature (ºC) was performed to check for primer-dimer formation within the samples.

Primer sequences for each gene investigated are provided in Table 2.1.

Table 2.1 – Primer sequences for measurement of inflammatory gene expression by q-PCR

	Forward Primer	Reverse Primer
iNOS	5' CAC ATT TGG CAA TGG AGA CTG C	5' GTT GGA AGT GTA GCG TTT CGG G
TNFα	5' CTA CTG AAC TTC GGG GTG ATC G	5' CAG TTG GTT GTC TTT GAG ATC C
EMR1	5' CTT TAC CAT CCT GGA TAA CAC C	5' ATG GAC ACA TTC TCC TCA TTG C
MCP-1	5' GCC TGT TGT TCA CAG TTG CTG C	5' GGG TCA AGT TCA CAT TCA AAG G
S9	5' TTG TCG CAA AAC CTA TGT CAC C	5' TTG CCT TCA AAC AGA CGC CG

Above table shows both forward and reverse primer sequences used to obtain cDNA and measure gene expression of the inflammatory and macrophage markers iNOS, TNFα, EMR1, and MCP-1 in the adipose tissue and skeletal muscle of rats by reverse transcription and real time PCR (qPCR). Ribosomal protein S9 was used as a housekeeper gene. Primers were sourced from GeneWorks, Australia.

2.3.4 Calculation of Relative Gene Expression

Quantification of mRNA expression for each gene was essentially as described previously [174]. Briefly, standard curves for each inflammatory marker and housekeeper gene were performed by purifying and cleaning PCR product for each marker, establishing a relative concentration of the cleaned product, and producing a serial dilution. The qPCR protocol was re-performed on the serial dilution of each marker. The standard curve was created by plotting the Ct values against log2 copies by linear regression and the following calculation (y=ax+b) could be determined. The coefficient of determination (R^2) of the line, and thus the standard curve, was used to calculate cDNA copy number in the original reverse transcription reaction, which is proportional to the initial mRNA concentration. This takes into account small differences in PCR amplification efficiency (ideally with a slope of 1 on the standard curve) that delta Ct comparisons used in some studies do not take into account. Gene expression was thus determined from the log of cDNA copy number determined from the Ct versus log copy number standard curve for each gene, and the absolute copy number expressed as a ratio to housekeeping gene (ribosomal S9 mRNA) copy number for each sample. This allowed to correct for differences in mRNA input into the PCR reactions. Note that this method preserves information about mRNA absolute copy number so that data from different PCR runs can be compared, whereas delta Ct comparisons must include samples from different experiments in a single run or establish that amplification efficiency has not changed between runs.

CHAPTER 3

SHORT-TERM DIET-INDUCED OBESITY AND INSULIN RESISTANCE IS NOT ASSOCIATED WITH INFLAMMATION IN RATS

3.1 Introduction

Dietary interventions in animal models have been used extensively to study a range of health outcomes. High fat feeding has proven to be an effective method to induce an obese phenotype in animal models and has been extensively used for research purposes to investigate diet-induced obesity and associated health implications such as insulin resistance and Type 2 Diabetes [26, 86, 138, 175]. Use of high fat diets mimics unhealthy eating habits that are prevalent in humans, with poor dietary intake being a leading cause of obesity and associated metabolic impairments. These animal models allow for a detailed examination of these impairments in order to gain further understanding into the mechanisms underlying their induction in addition to potential treatment avenues [71, 86, 176-178]. The vast majority of obesogenic diets are primarily based on a high fat content with most containing levels ranging from 20-60% of calories derived from fat [26, 86, 177, 179, 180]. Many variations of high fat dietary interventions exist, including the amount of fat content within the diet [86, 117, 138, 180], types of fat used [179, 181, 182], and duration of diet treatment [26, 117]. Some other dietary factors such as high sugar [183] or salt [184] have also been investigated in conjunction with high fat treatment.

High fat diets have been shown to be effective at producing significant obesity with associated insulin resistance after only short-term intervention in rodents. Approximately 4 weeks of high fat dietary intervention has been shown to induce significant obesity with associated insulin resistance in rats [86, 176, 180] in addition to also impairing microvascular recruitment and blood flow within skeletal muscle [86, 138]. Increased adiposity, impaired glucose tolerance, and systemic insulin resistance has been shown to develop after 3 days of high fat feeding in mice [26]. A number of inflammatory and macrophage markers are found to be significantly expressed within the adipose tissue in mice at this time-point, however the inflammatory response was shown to progressively worsen as high fat feeding extended long-term [26].

It has been widely reported that inflammation is associated with obese and insulin resistant conditions [18, 19, 26, 27, 123, 124, 185] however in spite of its strong association within the

literature, the rate by which inflammation develops in diet-induced obesity, and whether inflammation has the potential to impair microvascular and insulin-mediated responses at early stages of insulin resistance has not been fully established. A number of inflammatory markers have been highlighted as having a potential influence by impairing insulin sensitivity and vascular responses [122, 126, 127, 129]. Impaired insulin-mediated microvascular responses have been shown to be an early defect observed in insulin resistance as this altered blood flow response negatively impacts the delivery and uptake of glucose by the skeletal muscle [23, 32, 86, 138]. The vasculature may therefore be a critical site that inflammatory cytokines act at, and thus contribute to the development of whole body insulin resistance.

It has been shown that TNFα in rats can alter vascular responses and glucose uptake within insulin sensitive tissue such as skeletal muscle. Acute administration of TNFα in rats results in a significant inhibitory effect on insulin-mediated haemodynamic responses by blocking blood flow and capillary recruitment, resulting in an insulin resistant state with decreased glucose uptake by skeletal muscle and decreased whole body insulin sensitivity [126, 143]. There is also evidence that iNOS, the inducible isoform of nitric oxide synthase [108, 186], could be a contributing factor towards dysfunction in obese and insulin resistant states as shown in mouse studies [71, 127, 129]. This is believed to occur predominately due to increased vasoconstrictor responses of the vascular endothelium in order to compensate for the excessive production of nitric oxide when iNOS is induced which stimulated vasodilation of the vascular endothelium [127, 128, 161]. This results in significant endothelial dysfunction and alteration of vascular responses. Studies have also shown that iNOS knockout mice are protected against high fat diet-induced insulin resistance [71, 129]. Obese iNOS knockout mice display improved glucose tolerance and normal insulin sensitivity compared to obese wild-type mice after high fat feeding [129]. Whilst obese wild-type mice showed impaired activation of insulin signalling intermediates PI3K and Akt in muscle, obese iNOS knockout mice did not show these same defects [129]. Use of the nitric oxide inhibitor L-NAME has also shown similar metabolic improvements in mice [71, 129]. Preliminary studies conducted by Bussey (2011, University of Tasmania, PhD thesis) reported that rats fed a high fat diet for 4 weeks exhibited impaired vascular responsiveness to endothelin-1 in a perfused hindlimb model, and vascular responsiveness was restored to that seen in control diet-fed animals by the use of iNOS inhibitor 1400W, or the broader NOS inhibitor L-NAME. These findings suggested that even at early time points iNOS

overexpression potentially impairs vascular reactivity in skeletal muscle. The findings from these studies provide strong evidence that iNOS could play an important role in the development of insulin resistance.

Other cytokines such as MCP-1 are also strongly associated with obese conditions. MCP-1 strongly promotes and recruits macrophages to infiltrate into tissues of high fat fed mice, with its expression found to be significantly increased in obese adipose tissue [122]. These recruited macrophages may secrete a variety of cytokines and chemokines which can then promote an inflammatory response locally within the tissue [122]. And as mentioned above, a number of inflammatory markers have shown to have the ability to directly impair insulin's actions [126, 129]. Treatment of cultured human skeletal muscle cells with MCP-1 has shown impairment to insulin signalling and significantly reduced glucose uptake in the mycocytes [146]. Both insulin resistance and accumulation of macrophage markers within the adipose tissue were found to be extensively reduced in MCP-1 knockout mice compared to wild-type controls after high fat feeding [122]. These results suggest that MCP-1 may also have the potential to directly contribute towards the pathogenesis of insulin resistance in addition to its chemoattractant role in recruiting macrophages to initiate an inflammatory response.

Much research into the relationship between obesity and inflammation has been conducted in mouse models [26, 27, 123, 124, 177, 187], with similar studies in rats being severely outnumbered [185, 188]. The reasons for why mice are favoured over rats for inflammatory studies have not been greatly discussed within the literature. Mice, however, may potentially be preferred due to their small size and ease for genetic manipulation [26, 127, 129]. Many of the studies investigating inflammation and obesity have been conducted in specific knockout models [26, 127, 129], genetic models of obesity such as (ob/ob) mice [154, 189], as well as models which use chemicals to induce a diabetic and insulin resistant state such as streptozotocin [127, 190]. Although many studies have examined inflammatory expression in response to diet-induced obesity in mice [26, 71, 185, 191], the direct impact obesity-associated inflammation may have at impairing insulin-mediated responses have not been well established in rat models. Rats provide better models for studying larger system physiology due to both their larger size which enhances them as a disease model, and their

suitability for studying human diseases due to similar physiology [192]. They have been shown to be especially good models of cardiovascular disease, hypertension, and stroke [162, 192, 193]. These conditions are not only regularly associated with obesity, but have also been shown to have increased inflammatory responses [29, 100].

Because of this it is important to investigate inflammatory responses in rats to enhance and extend knowledge from mouse studies, but to also understand the relationship inflammation may have on larger physiological systems such as vascular responses and skeletal muscle insulin resistance. And while the majority of studies of obesity-induced inflammation have been reported from mice, the findings of Bussey (2011, Univeristy of Tasmania, PhD thesis) that iNOS overexpression potentially impairing vascular reactivity in skeletal muscle, suggested that a similar inflammatory process may occur in high fat-fed rats. As mentioned, mice are not amenable to study larger system physiology and the eventual measurement of inflammation-induced microvascular impairment for technical reasons. Currently contrast-enhanced ultrasound measurement of microvascular flow has not been performed for mice, and although a desirable objective, this would require considerable experimentation to perfect for mice, limiting the current thesis to the study of the microvascular effects of inflammation in rats, for which CEU has been extensively published [85, 87, 140, 194-197].

The fact that pro-inflammatory markers have been shown to impact haemodynamic responses and glucose uptake within skeletal muscle provides a preliminary theory as to how inflammation may contribute to altered metabolic responses seen in insulin resistance [126, 127, 129, 143]. This study aims to investigate whether inflammation occurs after a 4 week high fat dietary intervention in rats which provides an obese model with significantly impaired insulin-mediated responses such as stunted glucose uptake within skeletal muscle and whole body insulin resistance.

3.2 Materials and Methods

3.2.1 Animals

Male Sprague Dawley rats approximately 4 weeks of age were obtained from the University of Tasmania Central Animal Facility. On arrival rats were split into two groups with one group provided with a control diet (11% calories derived from fat), and the other a high fat (41% calories derived from fat) diet *ad libtitum* for 4 weeks. Drinking water was provided *ad libitum.*

Macronutrient composition of these diets are shown in Table 3.1.

Table 3.1 – Macronutrient composition of control and high fat diets expressed as % total weight

	CONTROL	HFD
Protein	19.4%	19.0%
Carbohydrate	70.7%	53.7%
Fat	4.8%	22.6%
Monounsaturated *(% total fat)*	39%	39%
Polyunsaturated *(% total fat)*	44%	9%
Saturated *(% total fat)*	17%	52%
Crude Fibre	5.1%	4.70%
Total Digestible Energy	14.0 MJ/Kg	19.9 MJ/Kg
Digestible Energy from Fat *(% total)*	**11%**	**41%**

3.2.2 Protocol

Surgery was performed as outlined previously in Chapter 2, section 2.2.1. Following the surgical procedure mean arterial blood pressure was allowed to stabilise for 1 hour, after which a 2 hour infusion of insulin (10 mU/min/kg) was initiated. A 30% glucose solution (wt./vol.) was infused at a variable rate to maintain basal blood glucose concentrations over the course of the experiment. Arterial blood glucose levels were measured every 10 minutes for the first hour, and every 15 minutes in the second hour of the clamp procedure using a glucose analyser (YSI 2300). Measurement of the blood glucose levels allowed the glucose infusion rate (GIR) to be adjusted accordingly to maintain basal levels. Figure 3.1 provides a detailed experimental protocol. Muscle glucose uptake and skeletal muscle microvascular perfusion were assessed as outlined in Chapter 2, section 2.2.2.

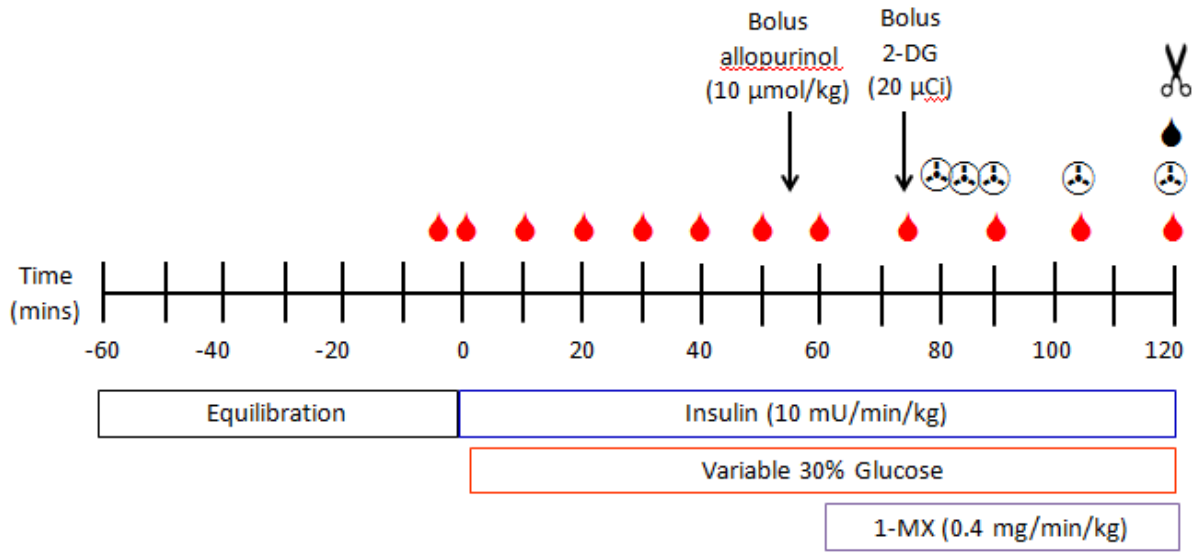

Figure 3.1 – Experimental protocol of hyperinsulinemic euglycaemic clamp procedure *in vivo*. Following surgical preparation a 60 minute equilibration period was allowed for stabilisation of blood pressure. Following this a continuous infusion of insulin (10 mU/min/kg) was commenced and continued for 120 minutes. A 30% (wt/vol) glucose infusion was initiated shortly after the commencement of the insulin infusion in order to maintain basal blood glucose levels. This was assessed by arterial blood sampling ( ). At 55 minutes a bolus of allopurinol (10µmol/kg) was given before an infusion of 1-methylxanthine (1-MX) was started at 60 minutes. At 75 minutes a bolus of 2-DG (20 µCi) was administered and radioactive plasma samples ( ) were collected at 80, 85, 90, 105, and 120 minutes to determine the clearance of plasma 2-DG. At the conclusion of the experiment arterial plasma samples were collected for the determination of hindlimb glucose uptake ( ). After samples were taken at 120 minutes animals were sacrificed and calf and epididymal fat pads were immediately excised, weighed (epididymal fat pads only) and freeze clamped in liquid nitrogen and stored at -80ºC.

3.2.3 Gene expression

Expression of inflammatory markers was performed by two step reverse transcription real-time PCR (RT-q-PCR). RNA was first extracted from skeletal muscle and adipose tissue samples and reverse transcribed using SuperScript III (Invitrogen) to obtain cDNA as described previously in Chapter 2, section 2.3. Real time PCR (qPCR) was performed using SYBR Green (Qiagen) to amplify the target DNA sequence and quantify gene expression of the chosen inflammatory markers (iNOS, TNFα, MCP-1, and EMR1) as described in Chapter 2, section 2.3. Inflammatory gene expression is shown as the relative expression to the housekeeper S9. The gene expression measurements were performed in a subset of study animals (5-6 per group) and powered to detect changes of 50% or greater.

3.2.4 Data and statistics

Data is present as means ± SEM and statistical analysis was performed using SigmaStat (Systat Software Inc). Comparisons between groups were made using un-paired Student's t-test and two-way ANOVA. Comparison of time-series measurements in each group was performed by two-way repeated measures ANOVA. When a significant difference of $p<0.05$ was detected, pairwise comparisons by Student-Newman-Keuls test was used to assess treatment differences.

3.3 Results

Four weeks of high fat feeding produced notable obesity with epididymal fat pad weight significantly heavier in high fat fed rats compared to controls (Figure 3.2, B). This adiposity was associated with significantly increased body weight of high fat fed rats (Figure 3.2, A).

Whole body insulin sensitivity was impaired in high fat fed rats compared to controls after 4 weeks of dietary intervention with high fat feeding. GIR during hyperinsulinemic euglycaemic clamps was significantly impaired in high fat fed animals compared to controls (Figure 3.3). Insulin stimulated significant glucose uptake in the skeletal muscle of both control and high fat fed rats compared to saline infusion (Table 3.2, Figure 3.4, A). However comparison of diet treatment between insulin clamps showed high fat fed rats to have a significantly blunted glucose uptake compared to control animals. Insulin also significantly increased microvascular perfusion within the skeletal muscle of control-fed animals but not in high fat fed rats (Figure 3.4, B).

The expression of pro-inflammatory and macrophage markers within the adipose tissue of high fat fed rats were found to be no different to control-fed animals after 4 weeks (Table 3.2). There were no significant correlations between inflammatory gene expression levels and GIR (data not shown).

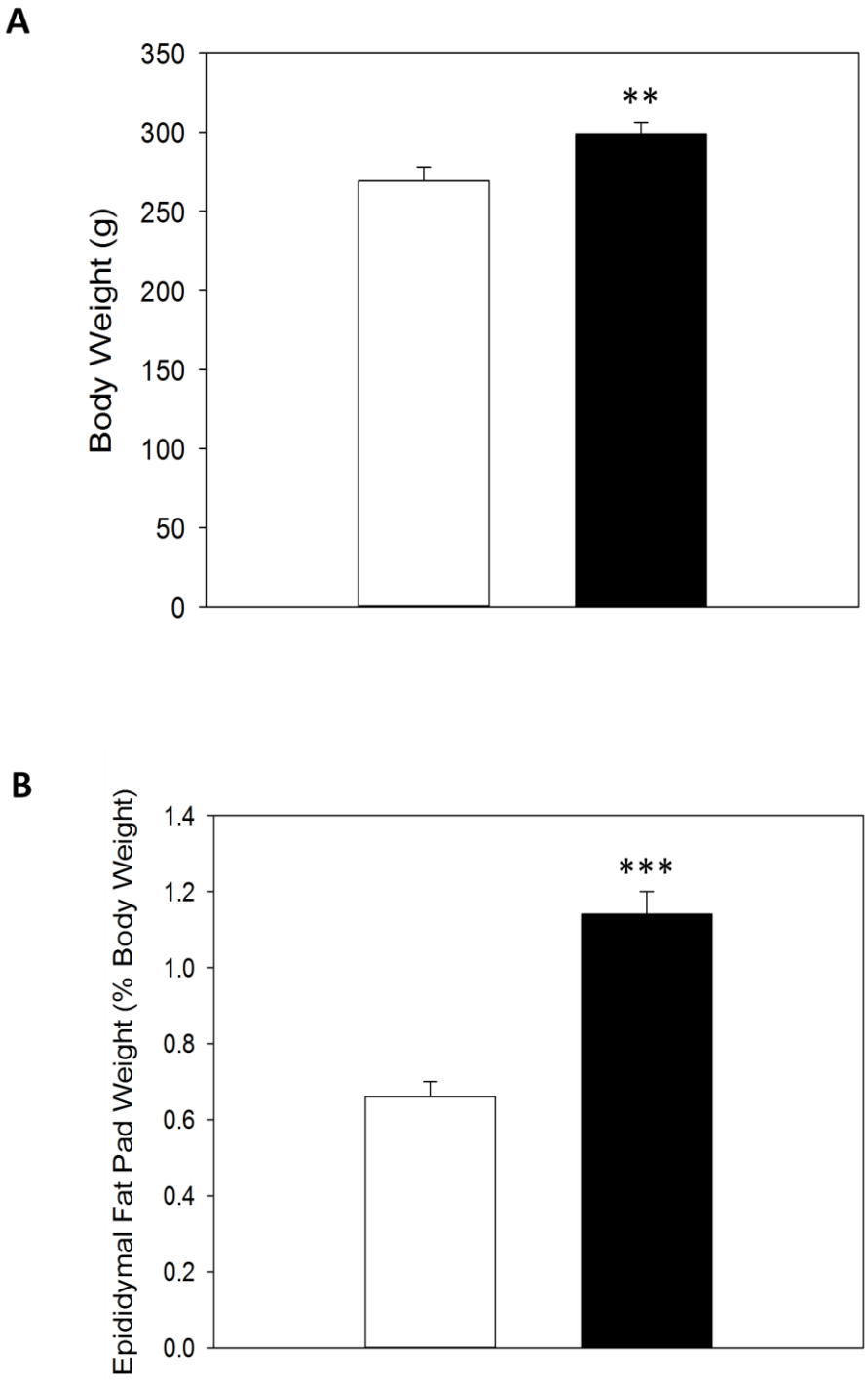

Figure 3.2 – Effect of 4 weeks high fat feeding (41% fat) on (A) body weight and (B) adiposity in control (□) and high fat fed (■) Sprague Dawley rats. Epididymal fat pads were excised and weighed immediately after removal. Mean wet weight as percentage of body weight is shown for each group ± SEM for n = 25-27 rats. ** = $p < 0.01$ and *** = $p < 0.001$ indicate significant difference by Student's t-test.

Table 3.2 - Effect of a 41% high fat diet on metabolic responses and insulin sensitivity of control and high fat fed rats.

	Control	HFD	Sig
Glucose Infusion Rate (mg/min/kg)	23.0 ± 0.2	18.0 ± 0.1	*P < 0.05*
Basal blood glucose (mmol/L)	3.6 ± 0.1	3.6 ± 0.1	NS
Muscle glucose uptake (R'g) (µg/g/min)	27.2 ± 1.6	15.7 ± 1.1	*P < 0.05*
Mean Arterial Pressure (mmHg)	111 ± 2	111 ± 2	NS

Metabolic parameters were measured by performing 10 mU hyperinsulinemic euglycaemic clamps in anaesthetised animals, with glucose infusion rate reflecting whole body insulin sensitivity. Muscle glucose uptake was calculated by 2-deoxy-D-Glucose uptake during final 45 minutes of clamp procedure. Data shows means ± SEM for n = 14-17 rats in each group. * = $p<0.05$ indicated significant difference between control and high fat diet groups for GIR measurement by two-way repeated measures ANOVA.

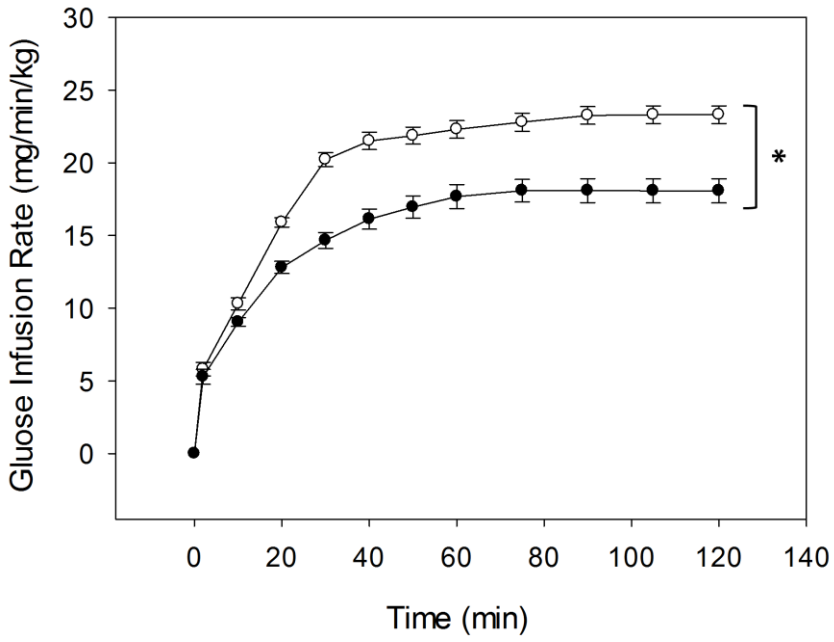

Figure 3.3 – Effect of a 41% high fat diet on whole body insulin sensitivity of control (○) and high fat fed rats (●) during hyperinsulinemic euglycaemic clamp. Data shows means ± SEM for n = 14-17 rats in each group. * = $p<0.05$ indicated significant difference between control and high fat diet groups two-way repeated measures ANOVA.

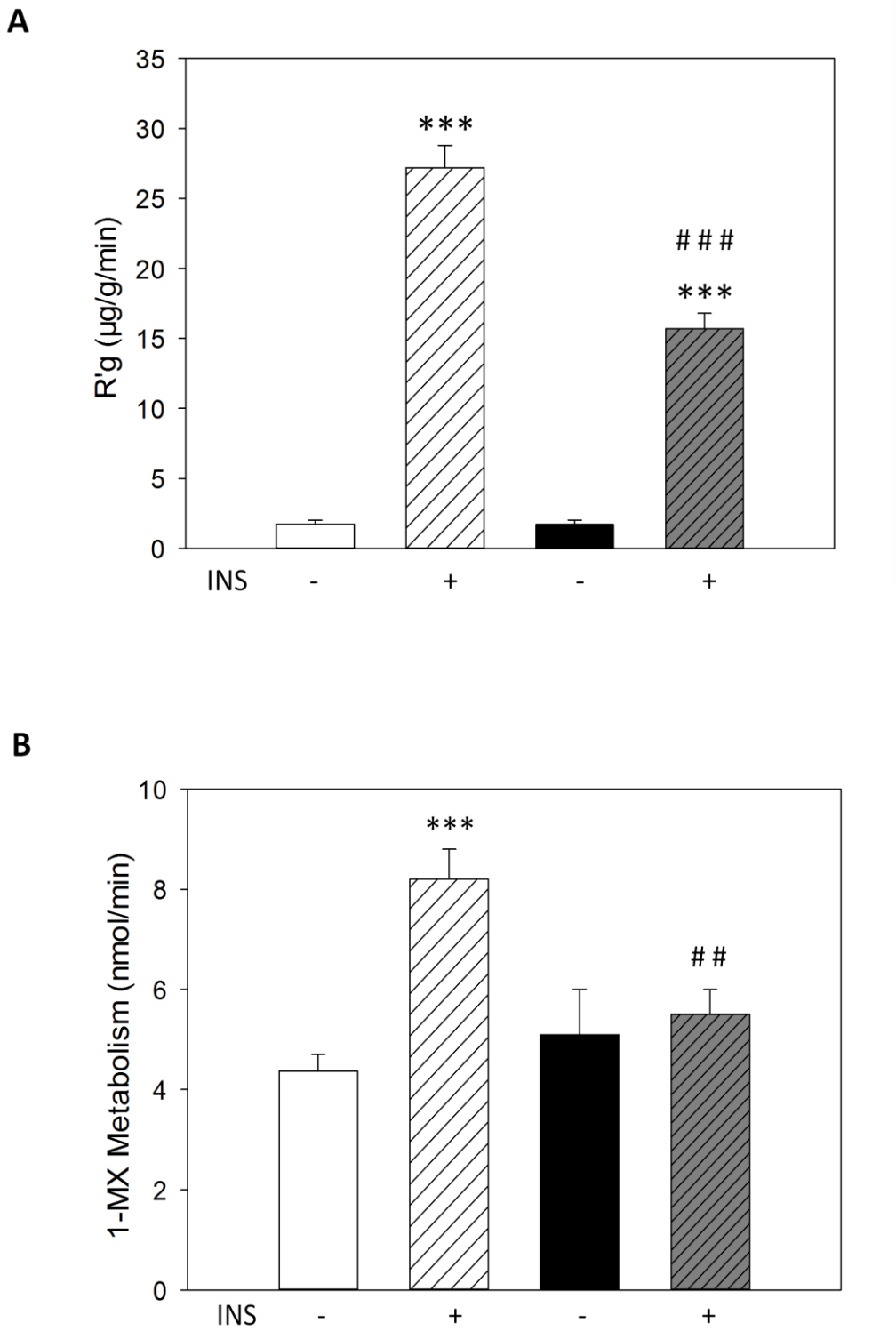

Figure 3.4 – Effect of a 41% fat diet on (A) muscle glucose uptake and (B) microvascular perfusion in the skeletal muscle control (□) and high fat fed rats (■) with or without insulin infusion during hyperinsulinemic euglycaemic clamp procedure. Muscle glucose uptake was calculated by 2-deoxy-D-Glucose uptake during final 45 minutes of clamp procedure. Microvascular perfusion within skeletal muscle was assessed by the metabolism of 1-MX. Data shows means ± SEM for n = 8-17 rats in each group. ** = $p<0.01$, *** = $p<0.001$ indicates significant difference within respective diet treatments; ## = $p<0.01$, ### = $p<0.001$ indicates significant difference from Control + Ins group by two-way ANOVA.

Table 3.3 – Comparison of inflammatory gene expression in visceral adipose tissue of control and high fat diet-fed rats after 4 weeks

EPIDIDYMAL FAT			
	Control	HF	*Sig*
iNOS	106 ± 44	164 ± 36	*NS*
TNFα	53 ± 11	35 ± 3	*NS*
EMR1	474 ± 72	635 ± 70	*NS*
MCP-1	1045 ± 331	1490 ± 383	*NS*

Table shows relative expression of inflammatory marker mRNA ratio to the housekeeper S9 in Sprague Dawley rats fed a control or 41% high fat diet for 4 weeks. Inflammatory gene expression was not found to be up-regulated in the visceral adipose tissue of high fat fed rats compared to controls. Data shows means ± SEM for n = 5-6 rats in each group. Statistical analysis was performed by Student's t-test.

3.4 Discussion

As expected and previously shown by other studies, 4 weeks of high fat feeding was effective at inducing an obese phenotype with associated insulin resistance in rats [86, 180, 198]. Use of a 41% fat diet produced significant adiposity in rats after only 4 weeks resulting in these animals being much heavier than matched controls (Figure 3.2). Whole body insulin sensitivity, as reflected by GIR, as well as glucose uptake and microvascular perfusion within skeletal muscle were all significantly impaired in rats fed this high fat diet compared to control-fed animals (Figure 3.3 & 3.4, Table 3.2). These results confirm high fat feeding to be an effective tool to study obesity and its associated metabolic complications.

However despite high fat feeding resulting in significant obesity and adipose tissue accumulation, expression of inflammatory and macrophage markers were not found to be significantly elevated within the adipose tissue of high fat fed rats compared to controls (Table 3.3). Only a subset of the cohort was examined in the inflammatory gene expression studies which may weaken the results, however these experiments were powered to detect a change of 50% or greater. Still the lack of any pro-inflammatory respose was surprising due to obesity-associated inflammation being regularly reported within the literature in other animal models with inflammation highlighted as an important factor in the development of obesity-associated insulin resistance [18, 27, 120, 199]. Although these rats showed significant adipose tissue accumulation, it is possible that a greater degree of obesity is required to initiate a significant pro-inflammatory response within the adipose tissue. Mice fed a high fat diet show excessive obesity with adipose tissue often reported to be two to three times greater than that of matched control animals after short-term feeding [26, 72, 200, 201]. Although our rats obtained significant adiposity with this 41% fat diet (Figure 3.2, B), the epididymal fat pad weight of rats on this diet showed approximately a 50% increase in adiposity compared to controls. Therefore the degree of obesity seen in our rats is not quite as large as that obtained in mice [27, 200, 202].

It is important to highlight that many high fat fed mouse studies use diets with a fat content where approximately 60% of the calories are derived from fat [26, 117, 120, 177, 201, 203].

In contrast the calories derived from fat in the diet used in this study equate to 41% (Table 3.1). Mice on this diet have been shown to produce significant adiposity that is approximately 3 times greater than that of control animals after only 2 weeks of dietary intervention [26]. Long-term studies in mice with this 60% high fat diet has also found significant inflammation within the liver, skeletal muscle, and adipose tissue after 16 weeks, however minor inflammation was measured in the adipose tissue within one week of high fat diet [26].

In conclusion, although a 41% high fat diet was shown to be successful at producing significant adiposity and insulin resistance in rats in did not induce a significant inflammatory response within the adipose tissue. It appears that obesity-associated inflammation may require a certain degree of obesity in order for an inflammatory response to be induced within the tissue. Increasing the dietary fat content should result in excessive lipid accumulation and added stress within the adipose tissue, which should result in the induction of macrophages and pro-inflammatory factors within the tissue. Long-term high fat feeding should also establish a chronic degree of obesity and insulin resistance and provide a more detailed analysis of the progression and development of obesity in rats, allowing for a more thorough relationship between obesity and inflammation over time.

CHAPTER 4

EFFECT OF FAT CONTENT AND DIET DURATION ON OBESITY, INSULIN RESISTANCE, AND INFLAMMATION

4.1 Introduction

As previously discussed in Chapters 1 and 3, high fat feeding has been used to study diet-induced obesity and its associated health implications such as insulin resistance and vascular dysfunction in animal models. High fat feeding has been widely reported as an effective method of increasing adiposity and inducing insulin resistance in rodents [26, 86, 202-205]. Although used widely, the exact composition of high fat diets used varies between studies [86, 138, 179, 180, 206]. Many obesogenic studies have favoured diets with a much higher fat content (60% calories from fat) than that used in Chapter 3 (41% calories derived from fat), despite diets with a lower fat content having been shown to be effective at inducing an obese and insulin resistant phenotype [138, 180, 182]. The majority of obesogenic studies report high fat diets have employed approximately 60% of the calories being derived from fat [26, 86, 120, 130, 176, 177, 198, 201, 203]. These higher fat diets have been shown to produce excessive obesity in mice with the adipose tissue of animals on this type of diet reported to be double or more of that measured in matched controls [26, 72, 200, 201, 207, 208]. The adiposity in high fat-fed rats appears more variable and may require much longer interventions to reach similar degrees of obesity to those seen in mice [86, 176, 198, 209-211]. Therefore it is important to directly investigate the use of a higher fat diet on adiposity in rats, and determine whether increased fat content, or a longer diet duration, results in a greater degree of adiposity (and inflammation) than that obtained previously in Chapter 3.

Both dietary fat content and diet duration could be important factors for the development of obesity and obesity-induced inflammation in rats. Long-term studies within the literature have regularly been reported as 8-16 weeks of intervention treatment, with some studies extending as far as 20 weeks or above [26, 117, 120, 191, 198, 202, 206, 212]. Long-term dietary interventions have shown that high fat feeding can produce significant obesity in animals, with adipose tissue accumulating to two to three times higher than that of age-matched controls. This is accompanied by significant insulin resistance and impaired glucose tolerance [26, 198, 202, 203]. These impairments have been shown to be induced in earlier stages of high fat feeding, however a number of long-term studies have found that they can become more severe as dietary intervention continues [26, 203]. The high fat diets used for

the majority of these long-term interventions have ranged between 45 – 60% energy from fat [26, 120, 191, 198, 202, 210].

As an inflammatory response was not seen in the adipose tissue of 4 week high fat fed rats (41% calories from fat, Chapter 3), using both a diet with a higher fat content (60% calories from fat) for 4 weeks and extending high fat feeding for a longer intervention (12 weeks) should allow for the development of significant obesity which may be more likely to induce an inflammatory response within the tissue. This dietary intervention should allow the degree of obesity required for significant inflammatory expression in high fat fed rats to be determined which will potentially provide further insight into the mechanisms required for the induction of inflammation. Use of a diet with a higher fat content requires greater lipid accumulation within the adipose tissue, as shown by the adipose tissue of animals fed this higher fat diet being double or more in weight compared to control animals [26, 86, 176, 201]. High fat fed mice have even shown significant adiposity as soon as 3 days after the commencement of a high fat diet [26]. Therefore this excess lipid accumulation places added stress upon the adipose tissue to respond and accommodate excessive lipid storage, even in a short period of time. The adipose tissue must undergo remodelling, often via hypertrophy or hyperplasia, in order to increase the storage capacity of the tissue in response to this demand [166]. This remodelling and additional stress may promote factors such as macrophages to migrate and proliferate within the tissue to induce an inflammatory response through the secretion of cytokines [122, 163, 166, 213]. Studies which have examined obesity-associated inflammation in mice record excessive adipose tissue obesity which is found to associated with the increased expression of a number of inflammatory factors within the tissue [26, 27, 123]. Inflammatory gene expression of certain factors such as TNFα, MCP-1, and EMR1 have been show to be induced within the adipose tissue as quickly as 3 to 7 days after high fat feeding, however a stronger and more significant inflammatory expression is observed as high fat feeding progresses long-term [26]. The majority of studies investigating obesity-associated inflammation have been conducted in mouse models, however very little is known about this response in rats.

A detailed time-course study investigating the differences between short and long-term high fat feeding with inflammation was conducted by Lee *et al.* in mice [26]. The expression of a

number of inflammatory markers, including TNFα, iNOS, MCP-1, and EMR1, was examined in the liver, skeletal muscle, and adipose tissue of mice that had undergone short-term (1, 3, and 7 days) and long-term (16 weeks) high fat feeding interventions. Although a few of these inflammatory markers showed significant increases at certain time-points during the short interventions, long-term high fat feeding produced a much more significant expression of all inflammatory genes in all tissue samples analysed. This expression ranged from 5 to 40 times that measured in controls. The study by Lee *et al.* [26] showed a clear systemic inflammatory response with long-term high fat feeding, however significant inflammation only occurred much later when obesity was well established in these animals. Despite this, the increases in inflammatory markers observed in both the adipose tissue and skeletal muscle of 16 week high fat fed mice [26] suggests that a similar systemic inflammatory response is possible in rats with long-term high fat feeding. However, comparable studies of obesity-associated inflammation, as that done by Lee *et al.* [26] in mice, have not been conducted in rats.

Therefore this study aims to investigate the expression of obesity-associated inflammation generated through dietary intervention with a significantly high fat diet (60% calories derived from fat) and longer exposure to the diet in rats. The use of both a higher fat diet and longer dietary intervention will allow for examination into the degree of adiposity required to induce an inflammatory response, and whether inflammatory factors generated from diet-induced obesity can further impair insulin sensitivity of high fat fed animals.

4.2 Materials and Methods

4.2.1 Animals

Male Sprague Dawley rats approximately 4 weeks of age were obtained from the University of Tasmania Central Animal Facility. On arrival rats were split into equal groups and provided with either a control (11% calories derived from fat) or high fat diet (58% calories derived from fat) *ad lititum* for 1, 4, 8 and 12 weeks. Macronutrient breakdown of these diets is shown in Table 4.1. Drinking water was provided *ad libitum*.

Table 4.1 – Macronutrient composition of control and high fat diets expressed as % total weight

	CONTROL	HFD
Protein	19.4%	19.4%
Carbohydrate	70.7%	39.9%
Fat	4.8%	36.0%
Monounsaturated *(% total fat)*	39%	34%
Polyunsaturated *(% total fat)*	44%	8%
Saturated *(% total fat)*	17%	58%
Crude Fibre	5.1%	4.70%
Total Digestible Energy	14.0 MJ/Kg	22.8 MJ/Kg
Digestible Energy from Fat *(% total)*	**11%**	**58%**

4.2.2 Protocol

Surgery was performed as outlined previously in Chapter 2, section 2.2.1. Following the surgical procedure mean arterial blood pressure was allowed to stabilise for 1 hour, after which a 2 hour infusion of insulin (10 mU/min/kg) was initiated. A 30% glucose solution (wt./vol.) was infused at a variable rate to maintain basal blood glucose concentrations over the course of the experiment. Arterial blood glucose levels were measured every 10 minutes for the first hour, and every 15 minutes in the second hour of the clamp procedure using a glucose analyser (YSI 2300). Measurement of the blood glucose levels allowed the glucose infusion rate (GIR) to be adjusted accordingly to maintain basal levels. Figure 4.1 provides a detailed experimental protocol. Muscle glucose uptake was assessed as outlined in Chapter 2, section 2.2.2.

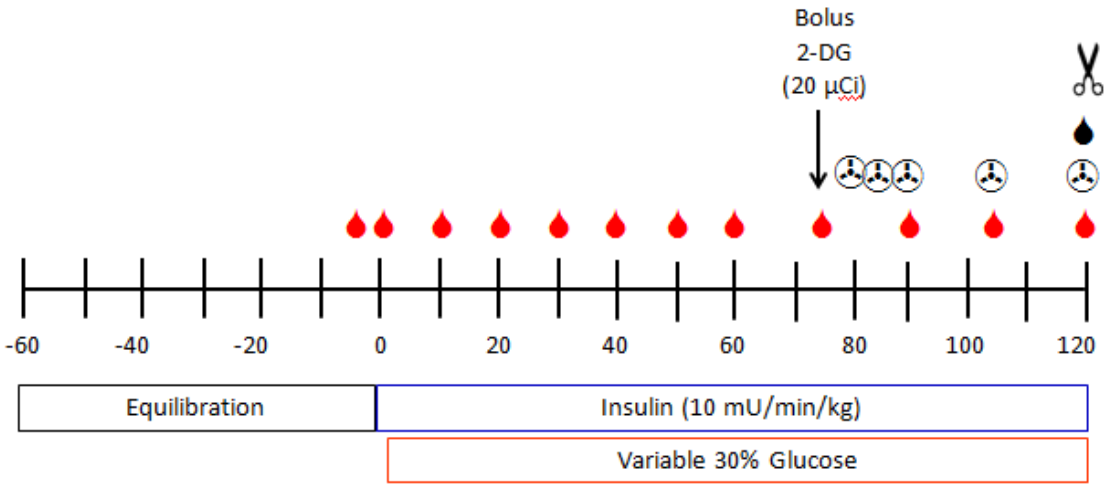

Figure 4.1 – Experimental protocol of hyperinsulinemic euglycaemic clamp procedure *in vivo*. Following surgical preparation a 60 minute equilibration period was allowed for stabilisation of blood pressure. Following this a continuous infusion of insulin (10 mU/min/kg) was commenced and continued for 120 minutes. A 30% (wt/vol) glucose infusion was initiated shortly after the commencement of the insulin infusion in order to maintain basal blood glucose levels. This was assessed by arterial blood sampling ( ). At 75 minutes a bolus of 2-DG (20 µCi) was administered and radioactive plasma samples ( ) were collected at 80, 85, 90, 105, and 120 minutes to determine the clearance of plasma 2-DG. At the conclusion of the experiment arterial plasma samples were collected for the determination of hindlimb glucose uptake ( ). After samples were taken at 120 minutes animals were sacrificed and calf and epididymal fat pads were immediately excised, weighed (epididymal fat pads only) and freeze clamped in liquid nitrogen and stored at -80ºC.

4.2.3 Gene expression

Expression of inflammatory markers was performed by two step reverse transcription real-time PCR (RT-q-PCR). RNA was first extracted from skeletal muscle and adipose tissue samples and reverse transcribed using SuperScript III (Invitrogen) to obtain cDNA as described previously in Chapter 2, section 2.3. Real time PCR (qPCR) was performed using SYBR Green (Qiagen) to amplify the target DNA sequence and quantify gene expression of the chosen inflammatory markers (iNOS, TNFα, MCP-1, and EMR1) as described in Chapter 2, section 2.3. Inflammatory gene expression is shown as the relative expression to the housekeeper S9. The gene expression measurements were powered to detect changes of 50% or greater.

4.2.4 Data and statistics

Data is present as means ± SEM and statistical analysis was performed using SigmaStat (Systat Software Inc). Comparisons between control and high fat fed rats were made using un-paired Student's t-test. Comparison of time-series measurements in each group was performed by two-way repeated measures ANOVA. When a significant difference of $p<0.05$ was detected, pairwise comparisons by Student-Newman-Keuls test was used to assess treatment differences.

4.3 Results

High fat feeding produced significant adiposity with short-term feeding interventions (1 and 4 weeks), with the epididymal fat pad mass being significantly increased in high fat fed rats compared to matched controls (Figure 4.2, B). With 1 week of high fat feeding this increased adiposity was reflected in a significant increase in body weight, however after 4 weeks of high fat feeding overall body weight was no different from control animals despite significantly increased adipose tissue mass (Figure 4.2, A). In comparison there is a clear lack of adiposity in long-term high fat fed rats (8 and 12 weeks) after dietary intervention with the epididymal fat pad mass of high fat fed rats being no different from matched controls (Figure 4.2, B). This lack of adipose tissue accumulation was also reflected by the body weight of long-term high fat fed rats also being no different from control animals for both 8 and 12 week time-points (Figure 4.2, A).

Whole body insulin sensitivity was not found to be impaired in high fat fed rats at either the 4 or 12 week time-points with this 58% high fat diet. Four week high fat fed rats showed no change in the rate of glucose infusion during the hyperinsulinemic euglycaemic clamps (Table 4.2, Figure 4.3, A) and glucose uptake within skeletal muscle was also found to be no different between high fat fed and control animals. Long-term high fat feeding produced no impairment of whole body insulin sensitivity during hyperinsulinemic euglycaemic clamps. In fact, these animals showed a significant increase of whole body insulin sensitivity measured by the clamp procedure compared to controls (Table 4.2, Figure 4.3, B). This increase in whole body insulin sensitivity was not associated with increased glucose uptake by the skeletal muscle (Table 4.2). Short-term high fat feeding (1 week) was also performed in older animals that were age-matched with the 12 week time-course (data not shown). Although 1 week of high fat feeding produced significant obesity in young animals the same effect did not occur in animals that were older (CON 1.71 ± 0.13 *vs* HFD 1.71 ± 0.09). These animals also showed intact insulin sensitivity and metabolic responses with GIR (CON 21.0 ± 0.2 *vs* HFD 21.6 ± 0.2) and skeletal muscle glucose uptake (CON 21.9 ± 1.5 *vs* HFD 20.9 ± 2.0) being no different from matched controls.

Pro-inflammatory and macrophage markers were not found to be significantly expressed in the adipose tissue or skeletal muscle of rats after short (4 week) or long-term (12 week) high fat feeding interventions compared to matched controls for any of the inflammatory markers analysed (Table 4.3, A-B).

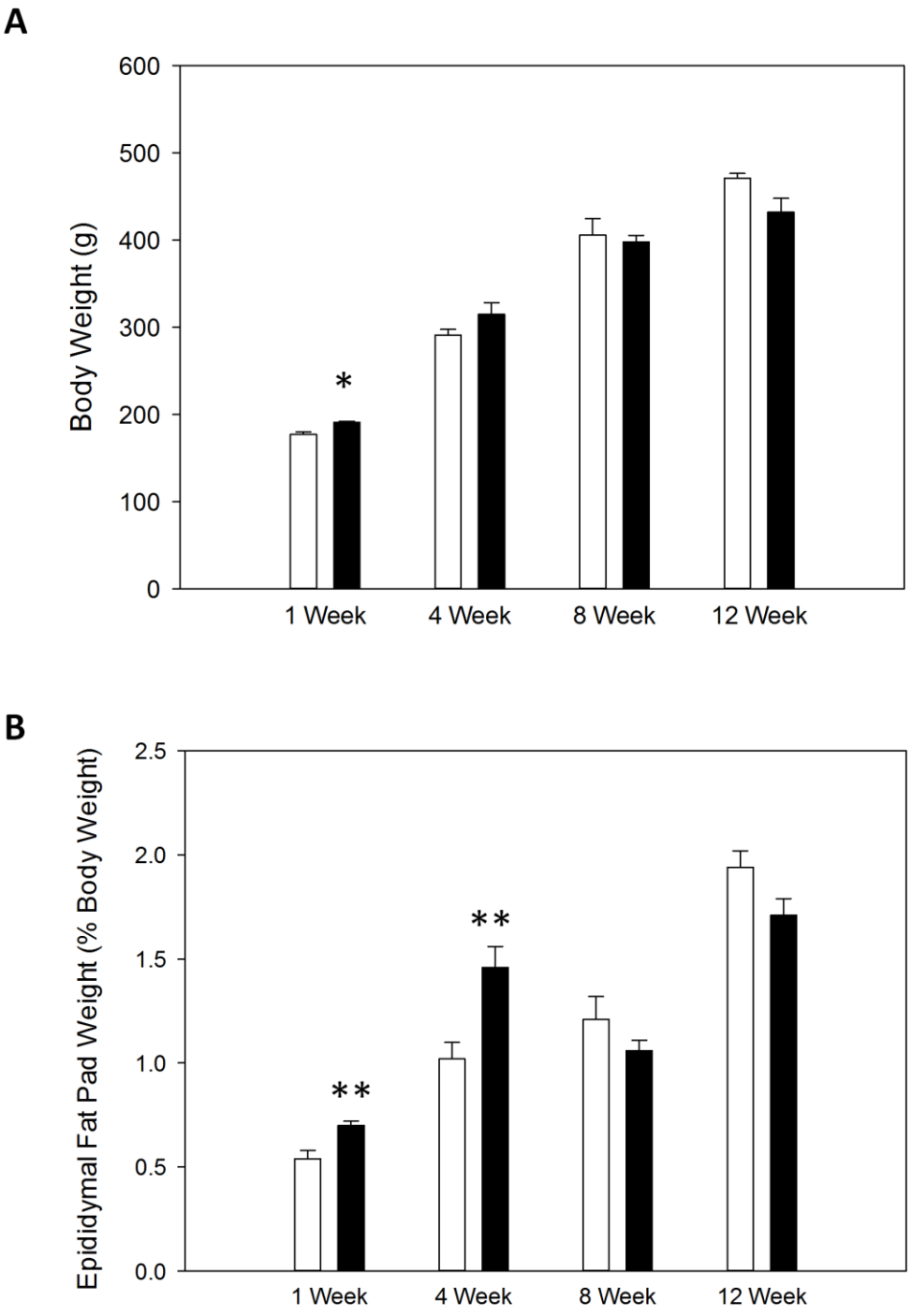

Figure 4.2 – Effect of a high fat diet (58% calories) on (A) weight gain and (B) adiposity over short and long-term interventions between Control (☐) and high fat fed (■) rats. Epididymal fat pads were excised and weighed immediate after removal and mean wet weight as percentage of body weight is shown for each group ± SEM for n = 5-9 rats with * = $p < 0.05$ and ** = $p < 0.01$ indicating significant difference from matched control animals by Student's t-test.

Table 4.2 - Metabolic responses and insulin sensitivity in short and long-term high fat fed rats using a 58% high fat diet.

	4 Week			12 Week		
	Control	HFD	*Sig*	Control	HFD	*Sig*
Glucose Infusion Rate (mg/min/kg)	23.6 ± 0.03	24.0 ± 0.10	NS	22.8 ± 0.2	29.9 ± 0.4	*P < 0.05*
Basal blood glucose (mmol/L)	4.4 ± 0.06	4.9 ± 0.2	NS	4.8 ± 0.2	5.7 ± 0.3	*P < 0.05*
Muscle glucose uptake (R'g) (µg/g/min)	10.4 ± 0.2	12.1 ± 0.9	NS	24.1 ± 3.2	31.1 ± 3.6	NS
Mean Arterial Pressure (mmHg)	111 ± 1	109 ± 2	NS	123 ± 2	118 ± 4	NS

Metabolic parameters were measured by performing 10 mU hyperinsulinemic euglycaemic clamps in anaesthetised animals, with glucose infusion rate reflecting whole body insulin sensitivity. Muscle glucose uptake was calculated by 2-deoxy-D-Glucose uptake during final 45 minutes of clamp procedure. Data shows means ± SEM for n = 8-9 rats in each group. Statistical analysis was performed by Student's t-test and GIR analysis was performined by two-way repeated measures ANOVA.

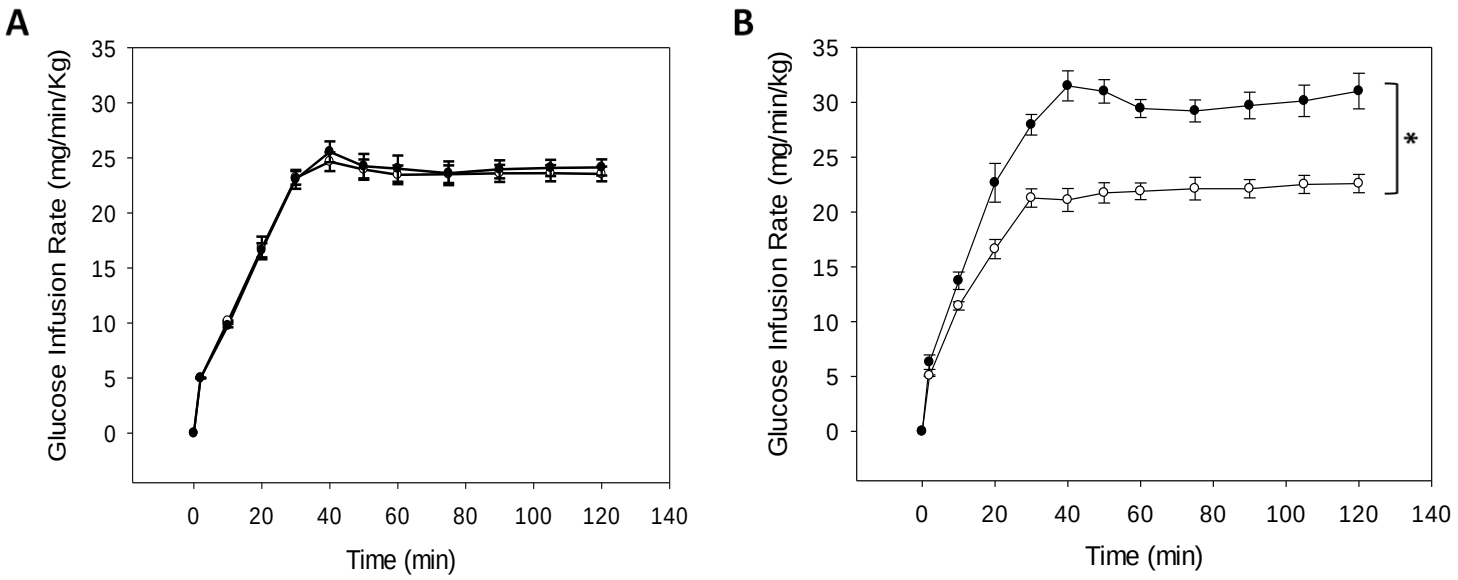

Figure 4.3 – Effect of high fat feeding (58% calories) on whole body insulin sensitivity in control (○) and high fat fed animals (●) by hyperinsulinemic euglycaemic clamp for (A) 4 week and (B) 12 week interventions. Data shows means ± SEM for n = 8-9 rats in each group. * = $p<0.05$ indicates statistical difference between control and HFD groups. Statistical analysis was performed using two-way repeated measures ANOVA.

Table 4.3 – Comparison of inflammatory gene expression in visceral adipose tissue and skeletal muscle of short and long-term high fat fed rats and controls

A

EPIDIDYMAL FAT	4 Week		12 Week	
	Control	HFD	Control	HFD
iNOS	106 ± 44	23 ± 7	57 ± 10	49 ± 4
TNFα	53 ± 12	16 ± 3	42 ± 4	27 ± 2
EMR1	474 ± 73	282 ± 22	448 ± 34	476 ± 101
MCP-1	822 ± 197	257 ± 20	1555 ± 119	997 ± 227

B

SKELETAL MUSCLE	4 Week		12 Week	
	Control	HFD	Control	HFD
iNOS	53 ± 3	54 ± 4	27 ± 3	36 ± 4
TNFα	9 ± 2	11 ± 2	7 ± 1	11 ± 3
EMR1	159 ± 14	134 ± 9	95 ± 11	103 ± 13
MCP-1	9 ± 2	7 ± 1	9 ± 2	9 ± 1

Inflammatory gene expression was measured by quantitative real time PCR (qPCR) and is shown as the relative expression to the housekeeper S9. An inflammatory response was not found to be expressed in (A) visceral adipose tissue or (B) skeletal muscle of both short (4 week) and long-term (12 week) high fat fed rats compared to matched control animals. Data shows means ± SEM for n = 8–9 rats in each group. Statistical analysis was performed using Student's t-test. Unless indicated, differences in individual marker expression between control and HFD at 4 and 12 week time-points are not significant.

4.4 Discussion

Although diets with very high fat contents have been widely reported to produce significant obesity and insulin resistance in rodents, the results in the current study showed that a diet in which 58% of the calories were derived from fat was ineffective at inducing an obese and insulin resistant phenotype in rats after both short and long-term dietary interventions. Rats fed the high fat diet showed significant adiposity after short-term dietary interventions (1 and 4 weeks), however longer interventions (8 and 12 weeks) showed that high fat fed rats were no more obese than control animals (Figure 4.2, B). Therefore the adiposity induced with short-term feeding was only transient and was not maintained or developed with longer intervention of this diet. Surprisingly insulin sensitivity measured by GIR during hyperinsulinemic euglycaemic clamp was found to be unchanged in 4 week high fat fed animals compared to controls, despite significantly increased adiposity (Table 4.2, Figure 4.3, A) unlike what has been previously reported within the literature by others which show significant impairments to insulin-mediated responses after 4 weeks [86, 176]. Unexpectedly, insulin sensitivity was found to be significantly increased in high fat fed rats after 12 weeks (Table 4.2, Figure 4.3, B), however the reasons for this response are unclear. Despite this response skeletal muscle glucose uptake in these animals was found to be no different from control animals (Table 4.2). The results obtained in this study were surprising as others have shown success with inducing an obese and insulin resistant phenotype in rats after similar high fat interventions [86, 176, 198, 211].

Comparison of the adiposity obtained after 4 weeks of high fat feeding was comparable to that obtained in similar studies in rats within the literature [86, 176]. However despite a similar degree of adiposity, insulin sensitivity and metabolic responses were not impaired in these animals but has been reported in other high fat studies. Studies conducted by St-Pierre using the same degree of high fat diet (58% calories derived from fat) and methods for measuring insulin sensitivity showed that 4 weeks of high fat dietary intervention is able to produce significant adiposity and impaired whole body insulin sensitivity in rats, and included impaired microvascular blood flow response in skeletal muscle [86, 176]. In particular skeletal muscle glucose uptake was found to be significantly blunted in high fat fed

rats (Δ -21% with 10 mU/min/kg insulin clamp) when compared to controls, and whole body insulin sensitivity was found to be significantly impaired in rats that had undergone 4 weeks of high fat feeding. GIR was found to be reduced after only 10 minutes of the 2 hour insulin clamp and this reduction was maintained for the duration of the clamp protocol [86]. It was expected that our rats would achieve a comparable degree of adiposity and a similar level of impaired insulin sensitivity after 4 weeks, and become progressively worse by 12 weeks. Therefore the lack of these impaired metabolic responses in our study was highly surprising. One of the main discrepancies between our study and those done by St-Pierre is the strain of rat breed used, with those by St-Pierre performed in Hooded Wistar rats [86, 176], whilst the current study was conducted in Sprague Dawleys. However, Sprague Dawley rats have also been reported to develop obesity and insulin resistance after high fat feeding with diets containing a lower fat content for 4 week interventions [138, 180]. It is therefore unclear as to why our rats did not produce similar metabolic impairment after intervention with a 58% high fat diet, but may be due to reduced palatability of the diet.

The inflammatory markers chosen for this study, TNFα, iNOS, and MCP-1, have been highlighted within the literature for their association with obese and insulin resistant states [122, 126, 127, 129]. These inflammatory markers have been shown to have the potential for impairing insulin sensitivity and altering vascular responses which may result in insulin mediated microvascular dysfunction [126-129, 143, 154, 187]. Significant inflammatory responses within the literature have been reported as large fold changes such as 2-3 fold increases or more of gene expression within tissues [26, 71, 124]. Although the lack of significant up-regulation of inflammatory gene expression in the tissues of high fat fed rats at either time-point was initially surprising (Table 4.3), the lack of well-developed obesity in these animals could explain why this response was not seen (Figure 4.2, B). Studies conducted in mice have shown that inflammation plays a role in more established and chronic insulin resistant and obese states while the initial onset of obesity is independent of an inflammatory response [26]. However, without an obese state, inflammation is unlikely to be observed. Inflammation is routinely reported in well-developed and chronic obese states [19, 26, 27, 188]. Therefore a model of diet-induced obesity with more overt adiposity than what was obtained would potentially provide better conditions for inflammation to be induced. It is possible that the higher fat content of this high fat diet is potentially not as appealing or as palatable as those containing less fat, and as a results may not be consumed in excess to

produce significant obesity, however measurement of food and calorie intake could confirm this [214].

This study has shown that a simple high fat feeding regimen in rats is not an effective method for producing a model of obesity-associated insulin resistance similar to that seen in humans. The reasons for this apparent variability in the outcomes of high fat feeding has not been widely identified or addressed within the literature. However some studies have identified that there are distinct variations in the phenotype induced through high fat feeding interventions [204, 215]. These varied outcomes from high fat feeding could potentially be due to high fat diets being classified as simply a 'high fat content' and not strictly standardised between studies [204]. Studies have shown that different dietary fats produce different responses in regards to weight gain and metabolic responses. Animal fats and plant oils high in omega 6 and omega 9 fatty acids have been shown to be effective at generating significant obesity and insulin resistance in rodents whereas animals fed fish oil and omega 3 fatty acids in high fat dietary studies did not develop these same responses [204, 215-217]. The type of dietary fat used in high fat feeding interventions is highly variable within the literature and some studies use diets with a combination of different fat sources [86, 179, 215, 218]. This variation of fatty acid compositions can therefore potentially produce varying results across studies especially in regards to weight gain and insulin sensitivity; therefore the type of high fat diet used in obesogenic dietary studies should be examined in detail to determine its suitability as a model of diet-induced metabolic dysfunction [204]. Comparison of the fat content of the control and high fat diets used in this study show that the high fat diet contains 36% fat wt/wt compared to the 4.8% fat wt/wt of the control diet. This equates to the digestible energy derived from fat being 58% for high fat and 11% for control diets (Table 4.1, Section 4.2). Breakdown of the dietary fat components into monounsaturated, polyunsaturated, and saturated fat species shows that the high fat diet contains a significant amount of saturated fat with more than half of the total fat content derived from saturated fat alone. Although diets high in fat have been shown to produce obesity and insulin resistance in humans and animals, high intake of saturated fats in particular have been associated with producing a more significant phenotype than high intake of other fat species [218, 219]. Therefore it remains surprising that long-term high fat feeding did not produce a significant response in our rats. However the composition of fat within the high fat diet may not be the

cause for the lack of adiposity and impaired insulin sensitivity in our animals and may be a result of other factors.

Another potential reason for the unsuccessful response to this diet could be its taste. The high fat content of the diet may not be palatable enough to stimulate excessive over-eating to result in significant adiposity, especially over longer dietary interventions [214, 220, 221]. Within this study, increased adiposity with high fat feeding was seen in younger animals on the diet for only short durations, while the long-term dietary intervention did not produce the same effect (Figure 4.2, B). As food and calorie intake was not measured in this experiment, the true palatability and consumption of the 58% high fat diet can only be hypothesised. It is possible that the high fat diet was initially well received and may be seen as a novel food choice compared to standard laboratory chow, however as the intervention continues this same diet may become monotonous and less palatable. A true obesogenic diet is therefore not always straightforward and a straight exchange of carbohydrate to fat may therefore not be an effective method for investigating diet-induced obesity and its associated health complications in all animal models. Studies have shown that rats exhibit food selection and have specific meal preferences with taste perception and flavour response strongly influencing diet consumption and over-eating [221-224]. Sensory factors have been identified as being equal to or more important than dietary composition in regards to food intake and excessive food consumption [225]. A high degree of adipose tissue accumulation is believed to be due to a combination of both excessive food consumption and poor dietary intake which includes highly processed foods, a high intake of bad fats, salt, and sugar [188, 212]. Therefore obesogenic diets should not always be associated with a straightforward calorie swap of one dietary component for another. Varied taste and dietary components such as fat and sugar have been shown to be more effective for inducing hyperphagia than each component on their own [226].

High fat feeding is routinely used to study diet-induced obesity in laboratory animals. This method of dietary interventions has been well reported within the literature and has been shown to produce significant obesity with impaired metabolic responses [117, 138, 176, 178]. However surprisingly, this study has shown that the effectiveness of high fat feeding is somewhat variable. Despite its reported success within the literature [26, 86, 117, 176], the

results from the current study found the use of a very high fat diet (58% calories derived from fat) to be less effective at inducing a model of obesity-associated insulin resistance than the lower fat content diet employed in Chapter 3, as our rats showed no impairment to insulin sensitivity, increased inflammatory expression, or significant and chronic obesity with both short and long-term interventions. The lack of significant or chronic obesity with this diet is the major limitation of this study and did not allow a study of the interaction between obesity, inflammation and insulin resistance. However the results highlight that high fat feeding is not always an effective way of producing an obese state with impaired metabolic responses. High fat diet treatments may only be more effective at one stage or timeframe and may only provide a short-term snapshot as opposed to long-term health complications of the obese state; as shown by the transient adiposity seen in rats fed this higher fat diet (Figure 4.2, B). Therefore care is needed when undertaking these types of investigations and factors such as dietary fat species, diet palatability, and variation should be taken into consideration [188, 215, 223]. Studies have shown that highly varied and palatable diets based on human snack food items, often referred to as the 'cafeteria-style diet', have been highly effective at inducing significant hyperphagia and obesity in rats [188, 220, 227, 228]. Further investigation into its ability to produce and maintain chronic obesity over long interventions would prove valuable in establishing its suitability as an alternative model to study the relationship between diet-induced obesity and inflammation, and the influence inflammation may have on metabolic dysfunction especially impaired vascular responses.

CHAPTER 5

CAFETERIA-STYLE DIET INDUCES SIGNIFICANT OBESITY AND INSULIN RESISTANCE, BUT NOT INFLAMMATION IN RATS

5.1 Introduction

Diets commonly used for obesogenic dietary studies often involve a simple exchange of calories derived from carbohydrate for those derived from fat [86, 205, 211]. Despite studies within the literature showing success with this method for inducing obesity, insulin resistance, and inflammation [26, 86, 138], experiments shown in Chapters 2 and 3 investigating high fat feeding over a range of time points have indicated that this is not always the case. It is important to note that this type of diet modification of simply swapping carbohydrate to fat is not a true representation nor does it provide the robustness of the poor diet habits adopted by humans that lead to obesity and its associated health complications [188, 229]. It has been shown that experimental diets consisting of a variety of energy dense and palatable snack foods, such as the cafeteria-style diet, are highly effective at producing a vast degree of obesity with significant metabolic impairment [188]. This type of diet is also a more accurate representation of poor food choices present in Western society where obesity has reached epidemic proportions.

The cafeteria-style diet consists of a variety of highly palatable and calorie dense food items often containing significant amounts of fat, sugar, and salt. Although the composition of this diet varies considerably between studies, food items with a high energy density are selected and these include snack food items such as peanut butter, cookies, cheese, condensed milk, cereals, and bacon [188, 220, 227, 230, 231]. The high palatability of these items in addition to a variety of different options on offer is believed to be what makes this diet effective at inducing hyperphagia and significant weight gain. Previous studies using this style of diet [188, 220, 227, 228, 232], have shown that rats given free access to these palatable foods produce excessive weight gain and dietary obesity with impaired metabolic responses. However despite its reported effectiveness in inducing obesity, the use of this cafeteria-style diet is highly variable within the literature, with both its availability (food items only given for specific time periods versus constant availability) and the number of food items (a few items interchanged daily versus all options available) on offer [220, 222, 227, 228].

The use of cafeteria diets proved a popular dietary intervention for investigating obesity in the 1980s [222, 232-235], however its use appeared to fall out of favour potentially due to commercial obeseogenic diets in pellet form becoming widely available for laboratory research which allows for the diet to consist of more purified and controlled components. Interestingly the use of cafeteria-style diets has seen somewhat of a resurgence in recent years [188, 220, 227, 228, 230, 236-238], however the exact reason for this has not been openly discussed in detail. It has been highlighted that foods high in sugar and fat are rewarding and are linked to binging episodes and addiction-like responses in animals [229, 237]. A large portion of recent studies using cafeteria-style diets have used them as a tool to investigate feeding patterns and behaviour in animals. Many of these studies focus on neurological responses such as appetite regulation, reinforcement, addiction-like behaviour, and have been associated with chronic over-consumption of palatable foods especially with extended interventions [220, 237-243]. The success of this cafeteria-style diet to induce significant hyperphagia and obesity allows for the mechanisms of feeding behaviour in relation to excessive nutrient intake and poor dietary health to be investigated within these studies. However despite a large amount of research being conducted in this area, cafeteria-style diets are still rarely used to study obesity-associated health complications such as inflammation and the metabolic syndrome [188, 230].

Sampey *et al* [188] has highlighted the fact that although a number of animal models examining dietary obesity exist, there is a lack of studies comparing these different diet treatments in rodents. Direct comparisons between different dietary interventions, such as commonly used high fat diets and cafeteria-style diet, have not been adequately examined or evaluated, and it has been alluded to that monotonous and rather bland laboratory diets – including high fat diets despite enhanced flavouring to improve palatability – may not reflect the normal regulation of feeding behaviour or degree of weight gain and metabolic responses that is possible in these animals compared to more varied and palatable diets. Therefore more comprehensive studies comparing different methods of diet-induced obesity are required in order to determine the effectiveness and suitability of dietary interventions for research purposes. For that reason this study aimed to compare the effectiveness of a cafeteria-style diet at inducing significant obesity to that obtained from high fat feeding, and whether the increased adiposity is able to be maintained over long periods of dietary intervention. If these

criteria were met, a second, more important aim (for this thesis) was to determine whether the increased adiposity was associated with inflammation in adipose tissue.

5.2 Materials and Methods

5.2.1 Animals

Male Sprague Dawley rats approximately 4 weeks of age were obtained from the University of Tasmania Central Animal Facility. On arrival rats were split into equal groups. One group was provided with a commercially available control diet (6% fat wt/wt) (Barrastoc), while the other was provided with control diet (6% fat wt/wt) and a 'cafeteria-style' diet consisting of two different human snack food items which were interchanged daily (Table 5.2). Of the snack foods chosen for the cafeteria-style diet, one high protein and one sweet or savoury item was selected for each day. Food items selected for this diet were based on studies that have used this cafeteria-style dietary intervention previously [188, 227, 230, 232]. All food items, along with drinking water, were available *ad libitum* for 4 and 12 weeks. All food items were purchased from Woolworths supermarket, Hobart, Tasmania, and were weighed daily to monitor food consumption.

Macronutrients in these diets are shown in Table 5.1.

Table 5.1 – Diet breakdown (% Calories) of control and cafeteria-style diets

	CONTROL	CAF
Protein	19%	15%
Carbohydrate	70%	37%
Fat	11%	48%

Table 5.2 – Food items used in cafeteria-style diet with macronutrient profile

	Calories (per 100g)	Protein (g)	Carbohydrate (g)	Fat (g)
High Protein				
Bacon	260	18	2	18
Peanut Butter	624	26	10	49
Pâté	336	9	2	33
Salami	428	5	1	9
Party Pies	225	8	22	12
Cheese Slices	276	20	20	48
Sweet/Savoury				
Sweet Biscuits	493	4	69	22
Donuts	328	6	49	12
Potato Chips	545	7	51	35
Cheesecake	306	5	36	16
Sugar Cereal (Froot Loops)	388	7	85	1
Condensed Milk	344	9	56	8
Banana Cake	342	5	52	13

5.2.2 Protocol

Surgery was performed as outlined previously in Chapter 2, section 2.2.1. Following the surgical procedure mean arterial blood pressure was allowed to stabilise for 1 hour, after which a 2 hour infusion of insulin (10 mU/min/kg) was initiated. A 30% glucose solution (wt./vol.) was infused at a variable rate to maintain basal blood glucose concentrations over the course of the experiment. Arterial blood glucose levels were measured every 10 minutes for the first hour, and every 15 minutes in the second hour of the clamp procedure using a glucose analyser (YSI 2300). Measurement of the blood glucose levels allowed the glucose infusion rate (GIR) to be adjusted accordingly to maintain basal levels. Figure 5.1 provides a detailed experimental protocol. Muscle glucose uptake was assessed as outlined in Chapter 2, section 2.2.2.

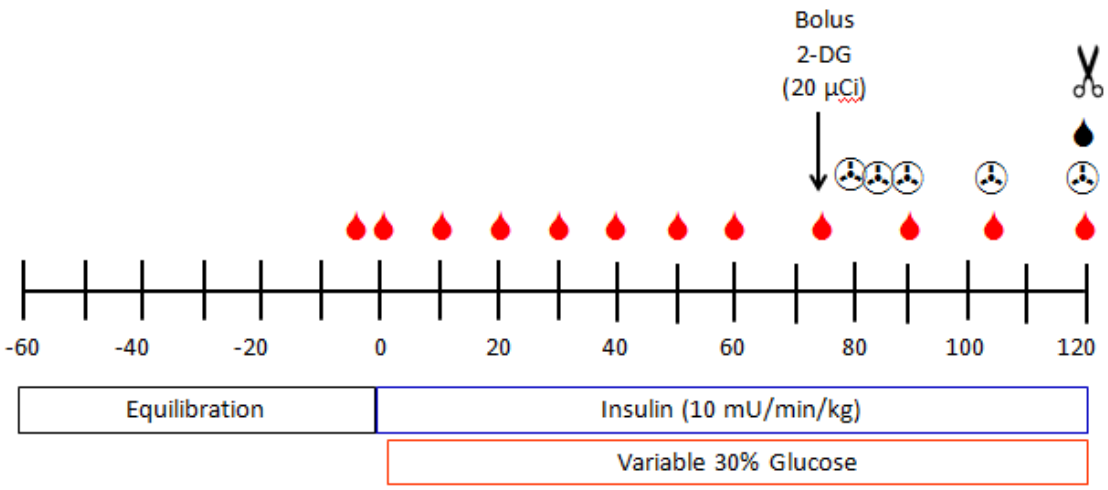

Figure 5.1 – Experimental protocol of hyperinsulinemic euglycaemic clamp procedure *in vivo*. Following surgical preparation a 60 minute equilibration period was allowed for stabilisation of blood pressure. Following this a continuous infusion of insulin (10 mU/min/kg) was commenced and continued for 120 minutes. A 30% (wt/vol) glucose infusion was initiated shortly after the commencement of the insulin infusion in order to maintain basal blood glucose levels. This was assessed by arterial blood sampling ( ). At 75 minutes a bolus of 2-DG (20 µCi) was administered and radioactive plasma samples ( ) were collected at 80, 85, 90, 105, and 120 minutes to determine the clearance of plasma 2-DG. At the conclusion of the experiment arterial plasma samples were collected for the determination of hindlimb glucose uptake ( ). After samples were taken at 120 minutes animals were sacrificed and calf and epididymal fat pads were immediately excised, weighed (epididymal fat pads only) and freeze clamped in liquid nitrogen and stored at -80ºC.

5.2.3 Gene expression

Expression of inflammatory markers was performed by two step reverse transcription real-time PCR (RT-q-PCR). RNA was first extracted from skeletal muscle and adipose tissue samples and reverse transcribed using SuperScript III (Invitrogen) to obtain cDNA as described previously in Chapter 2, section 2.3. Real time PCR (qPCR) was performed using SYBR Green (Qiagen) to amplify the target DNA sequence and quantify gene expression of the chosen inflammatory markers (iNOS, TNFα, MCP-1, and EMR1) as described in Chapter 2, section 2.3. Inflammatory gene expression is shown as the relative expression to the

housekeeper S9. The gene expression measurements were powered to detect changes of 50% or greater.

5.2.4 Data and statistics

Data is present as means ± SEM and statistical analysis was performed using SigmaStat (Systat Software Inc). Comparisons between control and high fat fed rats were made using un-paired Student's t-test. Comparison of time-series measurements in each group was performed by two-way repeated measures ANOVA. When a significant difference of $p<0.05$ was detected, pairwise comparisons by Student-Newman-Keuls test was used to assess treatment differences.

5.3 Results

Both short and long term interventions with the cafeteria-style diet produced overt obesity with epididymal fat pad mass being significantly heavier in cafeteria diet-fed animals compared to controls (Figure 5.1, B). This excessive fat accumulation also resulted in significantly elevated body weights of the cafeteria rats compared to control animals at both time points (Figure 5.1, A).

Whole body insulin sensitivity was found to be significantly impaired in the cafeteria diet-fed rats for both short and long-term interventions compared to matched controls, as reflected by significantly impaired GIR response during insulin clamps and blunted glucose uptake by the skeletal muscle within these animals (Figure 5.2).

Despite the presence of significant obesity and insulin resistance in rats fed this cafeteria-style diet, expression of pro-inflammatory and macrophage markers were surprisingly found to not be up-regulated in the adipose tissue after both short and long-term dietary interventions (Tables 5.1, A). Although a significant increase in the expression of iNOS was noted in the adipose tissue of long-term cafeteria diet-fed rats (Table 5.1, A), the lack of other pro-inflammatory or macrophage markers being up-regulated at the same time indicates that an inflammatory response was not occurring and that the increased expression of iNOS at this time point is potentially due to other un-related mechanisms. Furthermore, although the increase in iNOS expression at 12 weeks was significant, the change is quite small and confirms that this response is unlikely to reflect an inflammatory state. Due to inflammation not seen within the adipose tissue (Table 5.1, A), the lack of inflammation within skeletal muscle was not surprising (Table 5.1, B).

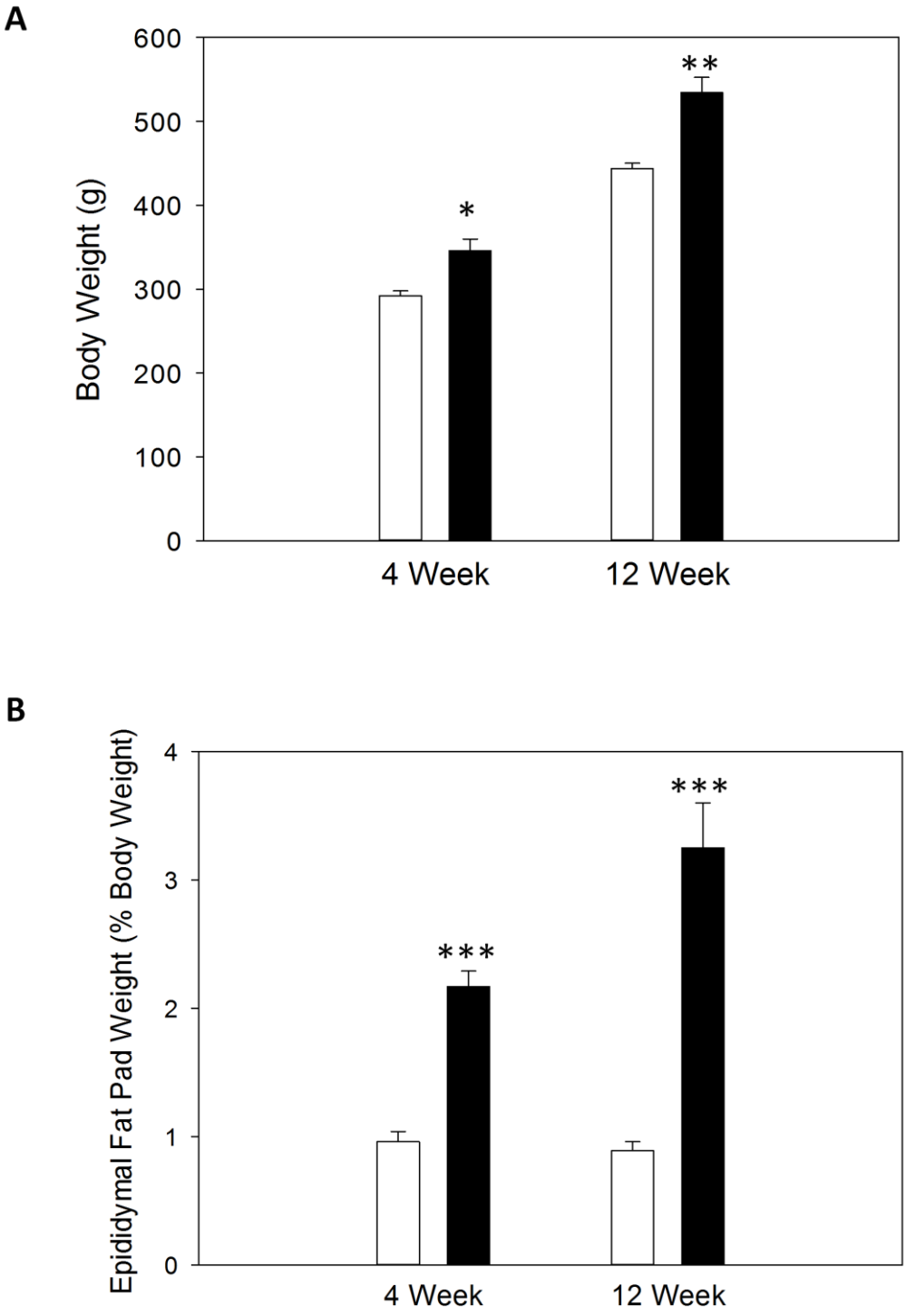

Figure 5.2 – Effect of a cafeteria-style diet on (A) body weight and (B) adiposity over short and long-term interventions between Control (☐) and cafeteria (■) diet-fed rats. Epididymal fat pads were excised and weighed immediate after removal and mean wet weight as percentage of body weight is shown for each group ± SEM for n = 6-8 rats with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ indicating significant difference from control animals by Students t-test.

Table 5.3 - Metabolic responses and insulin sensitivity in short and long-term cafeteria diet-fed rats.

	4 Week			12 Week		
	Control	HFD	*Sig*	Control	HFD	*Sig*
Glucose Infusion Rate (mg/min/kg)	26.0 ± 0.6	21.2 ± 1.3	***P = 0.001***	22.6 ± 0.1	16.7 ± 0.1	***P < 0.001***
Basal blood glucose (mmol/L)	4.4 ± 0.1	5.2 ± 0.3	***P < 0.05***	4.1 ± 0.1	4.4 ± 0.1	***P < 0.05***
Muscle glucose uptake (R'g) (µg/g/min)	9.1 ± 0.4	6.4 ± 0.5	***P < 0.01***	19.1 ± 1.1	15.7 ± 1.2	***P < 0.05***
Mean Arterial Pressure (mmHg)	115 ± 2	112 ± 2	*NS*	109 ± 2	109 ± 1	*NS*

Metabolic parameters were measured by performing 10 mU hyperinsulinemic euglycaemic clamps in anaesthetised animals, with glucose infusion rate reflecting whole body insulin sensitivity. Muscle glucose uptake was calculated by 2-deoxy-D-Glucose uptake during final 45 minutes of clamp procedure. Data shows means ± SEM for n = 6-8 rats in each group. Statistical analysis was performed by Student's t-test and GIR analysis was performined by two-way repeated measures ANOVA.

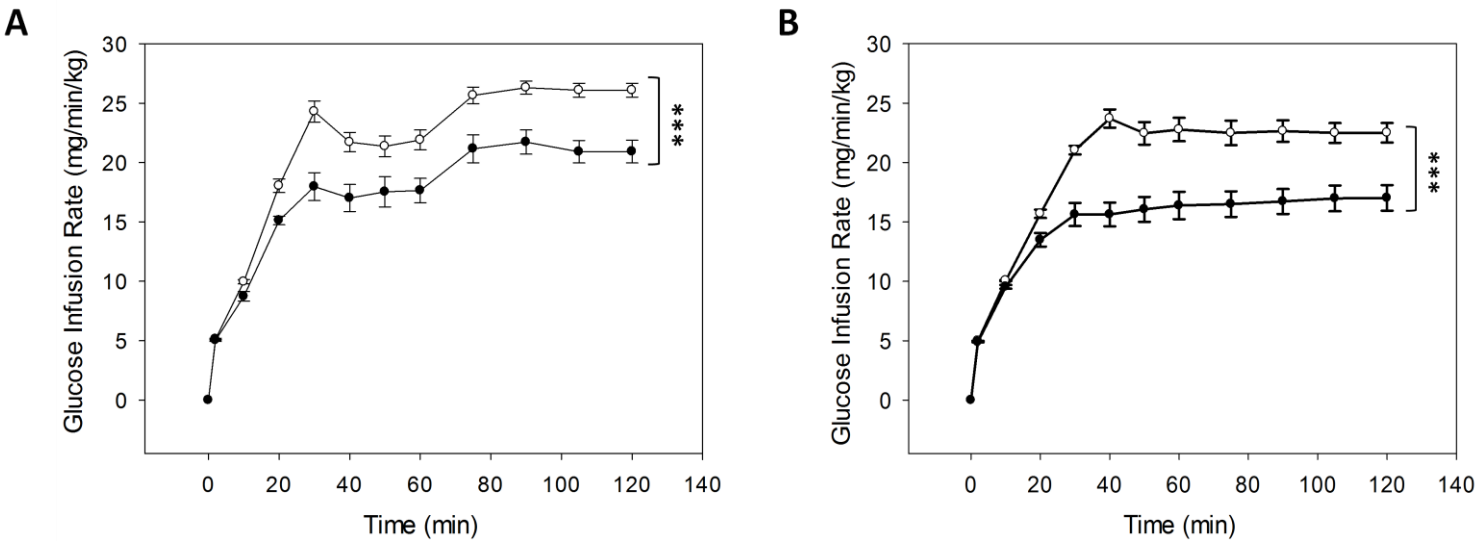

Figure 5.3 – Effect of cafeteria-style diet on whole body insulin sensitivity in control (○) and CAF animals (●) by hyperinsulinemic euglycaemic clamp for (A) 4 week and (B) 12 week interventions. Data shows means ± SEM for n = 6-8 rats in each group. *** = p<0.001 indicates statistical difference between control and CAF groups. Statistical analysis was performed using two-way repeated measures ANOVA.

Tables 5.4 – Comparison of inflammatory gene expression in adipose and skeletal muscle of cafeteria diet-fed rats and controls after short and long-term dietary intervention

A

EPIDIDYMAL FAT	4 Week		12 Week	
	Control	CAF	Control	CAF
iNOS	100 ± 45	39 ± 13	21 ± 6	76 ± 18 *
TNFα	40 ± 3	33 ± 7	34 ± 4	43 ± 4
EMR1	358 ± 50	378 ± 61	326 ± 34	181 ± 26
MCP-1	823 ± 197	257 ± 20	1252 ± 208	1492 ± 14

B

SKELETAL MUSCLE	4 Week		12 Week	
	Control	CAF	Control	CAF
iNOS	46 ± 8	53 ± 9	20 ± 3	34 ± 6
TNFα	6 ± 1	9 ± 1	10 ± 1	11 ± 2
EMR1	110 ± 7	129 ± 7	88 ± 4	86 ± 4
MCP-1	9 ± 4	6 ± 1	5 ± 1	6 ± 1

A pro-inflammatory response was not found to be expressed in (A) visceral adipose tissue or (B) skeletal muscle of rats fed a cafeteria-style diet compared to control animals for both 4 and 12 week dietary interventions. Data shows means ± SEM for n = 6-8 rats in each group. * indicates significant difference ($p<0.05$) from control animals by Students t-test. A significant increase was noted in the expression of iNOS in the adipose tissue of long-term cafeteria diet-fed rats (12 weeks) (A), however the lack of up-regulated expression of other inflammatory markers indicates the lack of a true inflammatory response occurring.

5.3 Discussion

The cafeteria-style diet was shown to be a highly effective tool for producing significant visceral adiposity with significant impairment of metabolic responses in rats that was sustained for 12 weeks. After 4 weeks of intervention epididymal fat pads were approximately double the weight of fat pads from control animals, however long-term cafeteria-diet intervention resulted in especially excessive obesity with epididymal fat pads being more than three times the weight of age-matched control diet-fed animals (Figure 5.2, B). Therefore not only was obesity maintained over the longer intervention but it became more developed as dietary intervention progressed. This obesity was also associated with the body weight of these animals being significantly increased compared to controls for both time-points (Figure 5.2, A). Cafeteria diet-fed rats also showed significant impairment of whole body insulin sensitivity and glucose uptake by the skeletal muscle followed both short and long-term dietary interventions (Table 5.3, Figure 5.3, A-B). Long-term cafeteria diet further reduced the GIR of cafeteria diet-fed rats compared to controls (Table 5.3, Figure 5.3, B).

Compared to previous results obtained with high fat diets (Chapters 3 and 4), the cafeteria-style diet has shown to be an effective tool for producing diet-induced obesity and insulin resistance in rats. The degree of adiposity in 4 week cafeteria diet-fed rats is almost double what was obtained with a 58% fat diet at 4 weeks, and is actually elevated about what was obtained after 12 weeks of high fat feeding. Looking at the adiposity of 12 week cafeteria diet rats shows that fat pad weight of these animals is more than double that of 12 week 58% fat rats (Chapter 4, Figure 4.2, B). This significant adiposity of cafeteria diet-fed rats was also associated with significant impairment to insulin sensitivity at both time-points, which was not seen in 58% high fat fed rats (Chapter 4, Table 4.2). Therefore these results emphasise that a more varied and palatable dietary intervention is significantly more effective than a diets based solely on increased fat content for producing an obese and insulin resistant phenotype in rats.

A number of rat studies have used significantly increased body weight as a measurement of obesity without directly reporting adipose tissue data [188, 220, 222, 227]. Although the significant increase in body weight of animals under dietary intervention can be presumed to be due to increased fat accumulation, it has not been quantified. As shown in this study, although the body weights of cafeteria diet-fed rats were significantly heavier than controls at both 4 and 12 week time-points, their adiposity was even more significant (Figure 4.2, B). In studies that report adipose tissue measurements, the degree of adiposity after 4 to 8 weeks of a cafeteria-style diet is comparable to that achieved in the current 4 week time-course with rats showing approximately a doubling of adipose tissue weight compared to control animals [244-246]. Long-term studies in mice (approximately 12 to 15 weeks) have shown an increase of adiposity 3 to 4 times that of control animals [247, 248]. This is comparable to the adiposity observed in the 12 week cafeteria diet-fed rats (Figure 4.2, B). That is, the cafeteria diet, but not the high fat diet, can achieve a similar degree of adiposity as obese mice in which inflammation is evident.

Although the cafeteria-style diet produces a robust model of diet-induced obesity, it can create difficulties within a research setting. While the cafeteria diet is loosely defined as the feeding of Western-style snack food items, the fact is that this diet is not standardised and highly varied diet compositions have been reported within the literature [188, 207, 220, 227, 228, 230, 232, 237]. This can complicate comparisons between different cafeteria diet studies in contrast to more reproducible commercially available laboratory diets. Although the cafeteria diet is successful at inducing significant obesity in both rats and mice the degree of adiposity reported is somewhat variable. Some studies have reported a doubling of adipose tissue mass whilst others report it to be 3 or 4 times higher than controls [244-249]. Reasons for these differences could include the variable nature of the cafeteria-style diets reported in the literature [188, 207, 220, 227, 230, 232, 249]. Accurate recording of food intake during dietary interventions can be challenging, however the cafeteria-style diet can introduce added complexity or error due to the range of different food items on offer. The nature of certain food items often selected include biscuits, cakes and potato chips to which are prone to crumble, spillage of liquids such as condensed milk, and the desiccation of certain foods such as meats and cheese provides sources of error that do not occur with low water content laboratory chows [227]. Despite this, the effectiveness of the cafeteria-style

diet has shown that both the diet palatability and variability are important factors to consider in regards to developing significant diet-induced obesity [214, 220, 223].

Both results from the dietary studies in this thesis and research done by others have shown that palatability and variety contribute towards inducing hyperphagia and overt obesity [188, 220, 223, 227]. It is important to note that some commercial diets alter their palatability and include the addition of flavouring to enhance taste response and reception in research animals [224, 225]. Therefore comparison of high fat fed animals to those fed a cafeteria-style diet shows that diet variability in particular may determine food intake and hyperphagia in these animals. A number of studies have investigated the feeding behaviour of rats and have found that rats consume more when multiple food items of different tastes are offered compared to when only a single food choice is given [221-224, 250]. Over-eating in rats on this varied diet appears to occur due to multiple food items being sampled which as a result increases the size of the meals consumed during each feeding period [222]. Therefore it has been hypothesised that satiety in rats is sensory-specific and that animals on a cafeteria or varied diet have excessive food consumption due to a delayed satiety response [225].

The most surprising finding to come from this study was that even with overt obesity and significantly impaired insulin sensitivity, cafeteria diet-fed rats lacked a pro-inflammatory response within insulin sensitive tissues, with the lack of inflammation within the visceral adipose tissue being especially unexpected (Table 5.4, A-B). Because of the increased adiposity resulting from this diet, an up-regulation of inflammatory and macrophage markers was anticipated as inflammation has been widely reported to be present in obese conditions and insulin resistance within the literature [18, 19, 26, 27, 199]. However findings from this study have shown that inflammation is not significantly expressed in rats despite significant obesity and insulin resistance. Therefore it can be stated that obesity and insulin resistance can occur independent of inflammation, and that inflammation is not a prerequisite driving factor in the development of impaired metabolic responses in obese states. Interestingly the majority of studies examining obesity-related inflammation have been conducted in mouse models [26, 27, 72, 117, 123, 124, 129, 170, 177, 189, 191, 200, 208, 251-253] and greatly outnumber similar studies in rats [185, 188, 210, 211, 231]. The exact reason for this has not been discussed within the literature, however the results from this study leads us to

hypothesise that rats possess mechanisms protecting them against developing significant inflammatory responses under these conditions of dietary intervention.

A potential explanation for the lack of an inflammatory response is the ability of adipose tissue to exhibit different growth responses during increases in lipid storage. Healthy adipose tissue expansion with adequate blood flow and oxygen availability despite excessive lipid accumulation is not associated with an inflammatory response, however inflammation has been strongly associated with adipose tissue dysfunction during unhealthy tissue expansion. As stated previously, the exact signalling pathways and mechanisms required for both healthy and unhealthy adipose tissue expansion are not well understood, and other additional mechanisms including genetic factors, genetic predisposition, and even the gut microbiome could be mediators or contributors to this regulation. Therefore further research into these mechanisms will allow for a better understanding as to the full potential of adipose tissue in a number of health complications and disease.

CHAPTER 6

METFORMIN RESTORES SKELETAL MUSCLE INSULIN SENSITIVITY AND MICROVASCULAR RECRUITMENT IN INSULIN RESISTANT RATS

6.1 Introduction

Obesity and insulin resistance are significant causes of health problems worldwide due to their link to cardiovascular disease and Type 2 Diabetes [116, 254]. Effective treatment that improves or restores insulin sensitivity aims to significantly reduce the morbidity and mortality that can occur without intervention. Lifestyle modifications such as diet, exercise, and weight loss remain the most beneficial treatment options for obese and insulin resistant individuals. However drug therapy is also required for patients that are unable to effectively implement lifestyle modifications such as exercise and weight loss. In addition to its ease of use drug therapy has more rapid results for controlling symptoms such as hyperglycaemia [12, 137, 255-257]. Preferably medication is used in conjunction with lifestyle modifications to obtain the most beneficial outcome for patient health.

Because insulin resistance is a key risk factor for the development of Type 2 Diabetes, a number of therapeutic agents have the primary action of improving insulin sensitivity [258-260]. There are two main classes of insulin sensitising drugs used for the treatment of insulin resistance. These include the biguanide and thiazolidinediones groups, with metformin and rosiglitazone being the primary drugs in each group respectively [258]. However despite their use, the mechanisms of these agents are not understood in detail [254, 258]. Thiazolidinedione treatment, such as the use of rosiglitazone, has been shown to improve insulin sensitivity by stimulating the uptake of glucose by skeletal muscle [261-263]. Although this family of drugs have proven to be effective at improving hyperglycaemia, their use has become controversial due to their association with increased risk of cardiovascular complications such as heart failure and stroke [264, 265].

In comparison, metformin has shown significant success with improving insulin sensitivity within insulin resistant individuals, and is currently the preferred drug treatment available for the regulation and control of blood glucose levels, having few adverse side effects [260, 265]. From the biguanide family, metformin is now the preferred drug for the treatment of type 2 diabetes [255, 266]. It is highly effective at reducing blood glucose levels, primarily through

the suppression of hepatic glucose production by the liver [267, 268], and it has also been shown to increase skeletal muscle glucose uptake [269, 270] however this action is still somewhat contentious [259, 266]. In addition to its insulin sensitising actions, metformin also significantly lowers the risk of cardiovascular-related death, such as by heart attack and heart failure [255, 271]. Cardiovascular disease is one of the leading causes of mortality in insulin resistance and type 2 diabetes [272]. Therefore metformin's ability to effectively reduce these events, in addition to its ability to improve insulin sensitivity, is one of the primary reasons it has become the preferred treatment option.

A number of studies have investigated the effect of metformin treatment on the incidence of cardiovascular events, such as myocardial infarction, heart failure, and stroke, in human subjects, which associate with macrovascular dysfunction [273, 274]. Many studies which have investigated metformin's ability to alter vascular responses have done so in regards to endothelium-dependent and endothelium-independent vasodilation, often by the use of acetylcholine and sodium nitroprusside respectively [272, 275, 276]. Although these studies have provided insight into some of the mechanisms surrounding vascular dysfunction, few studies have been conducted to investigate metformin's ability to alter microvascular responses such as capillary recruitment within insulin resistant skeletal muscle *in vivo*. Under normal conditions insulin recruits additional blood flow within the microvasculature of muscle to allow for increased substrate and glucose delivery to the skeletal myocytes [23]. However impaired capillary recruitment and blood flow within the microvascular has been identified as a defect leading to the early development of insulin resistance in muscle [86]. As metformin has been shown to have some vascular action in addition to significantly improving hyperglycaemia and insulin sensitivity in insulin resistant individuals, it is possible that it also acts upon the microvasculature to improve the delivery of glucose to muscle via the recruitment of capillary blood flow.

What is unclear about metformin's actions in insulin resistance is whether it improves muscle insulin sensitivity as a result (at least in part) of improved microvascular perfusion, and whether this occurs by opposing the effects of inflammation, as opposed to affecting other factors that impair insulin signalling such as altered lipid metabolism and distribution.

Metformin has been reported to have anti-inflammatory effects and these occur irrespective of diabetes status [277]. Although initially hypothesised in this thesis that diet-induced obesity would be accompanied by inflammation, its repeated absence offers an opportunity to establish whether metformin's insulin sensitising actions in muscle (i) involve improved muscle microvascular blood flow and (ii) results from an anti-inflammatory action or not. An absence of improved insulin sensitivity with metformin treatment would then suggest that its anti-inflammatory action is critical. Thus the aim of this chapter was to investigate whether the loss of whole body muscle and microvascular insulin sensitivity due to diet-induced obesity could be restored by metformin treatment.

6.2 Materials and Methods

6.2.1 Animals

Male Sprague Dawley rats approximately 4 weeks of age were obtained from the University of Tasmania Central Animal Facility. On arrival rats were split into equal groups with all rats receiving a high fat diet (41% calories derived from fat) *ad libtitum* for 4 weeks.

One of the high fat diet groups was also treated with metformin for the final 2 weeks of dietary intervention. Metformin was given at a dose of 150mg/kg/day in the drinking water which was monitored daily and changed every 2 to 3 days. All drinking water, with or without metformin, was provided *ad libitum*.

Macronutrient content of the 41% high fat diet in comparison to a control diet is shown in Table 6.1.

Table 6.1 – Macronutrient composition of control and high fat diet diets expressed as % total weight

	CONTROL	HFD
Protein	19.4%	19.0%
Carbohydrate	70.7%	53.7%
Fat	4.8%	22.6%
Monounsaturated *(% total fat)*	39%	39%
Polyunsaturated *(% total fat)*	44%	9%
Saturated *(% total fat)*	17%	52%
Crude Fibre	5.1%	4.70%
Total Digestible Energy	14.0 MJ/Kg	19.9 MJ/Kg
Digestible Energy from Fat *(% total)*	**11%**	**41%**

6.2.2 Protocol

Surgery was performed as outlined previously in Chapter 2, section 2.2.1. Following the surgical procedure mean arterial blood pressure was allowed to stabilise for 1 hour, after which a 2 hour infusion of insulin (10 mU/min/kg) was initiated. A 30% glucose solution (wt./vol.) was infused at a variable rate to maintain basal blood glucose concentrations over the course of the experiment. Arterial blood glucose levels were measured every 10 minutes for the first hour, and every 15 minutes in the second hour of the clamp procedure using a glucose analyser (YSI 2300). Measurement of the blood glucose levels allowed the glucose infusion rate (GIR) to be adjusted accordingly to maintain basal levels. Figure 6.1 provides a detailed experimental protocol. Muscle glucose uptake and skeletal muscle microvascular perfusion were assessed as outlined in Chapter 2, section 2.2.2.

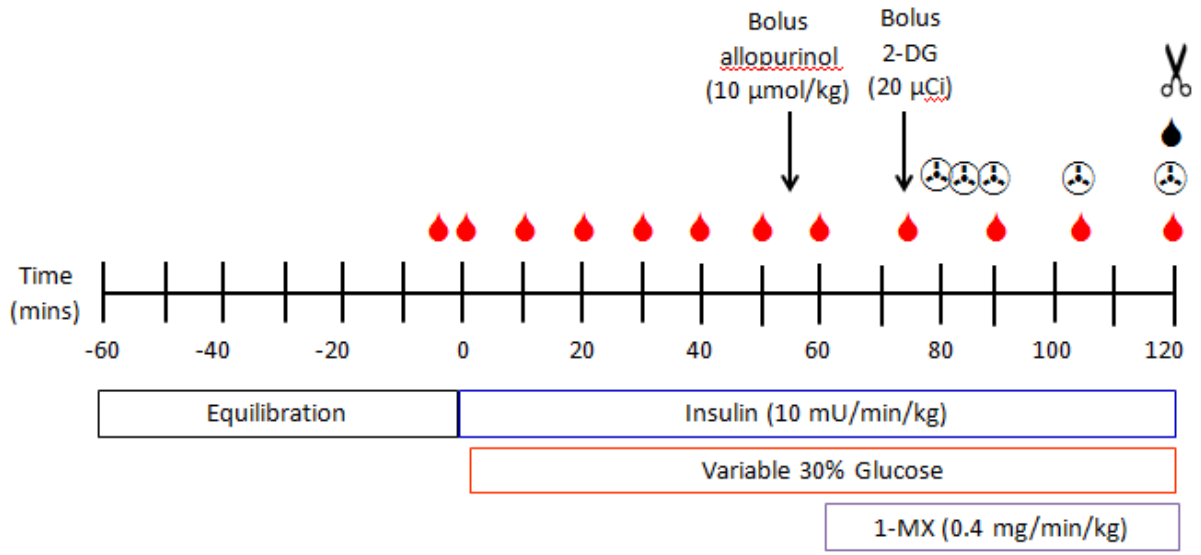

Figure 6.1 – Experimental protocol of hyperinsulinemic euglycaemic clamp procedure *in vivo*. Following surgical preparation a 60 minute equilibration period was allowed for stabilisation of blood pressure. Following this a continuous infusion of insulin (10 mU/min/kg) was commenced and continued for 120 minutes. A 30% (wt/vol) glucose infusion was initiated shortly after the commencement of the insulin infusion in order to maintain basal blood glucose levels. This was assessed by arterial blood sampling ( ). At 55 minutes a bolus of allopurinol (10µmol/kg) was given before an infusion of 1-methylxanthine (1-MX) was started at 60 minutes. At 75 minutes a bolus of 2-DG (20 µCi) was administered and radioactive plasma samples ( ) were collected at 80, 85, 90, 105, and 120 minutes to determine the clearance of plasma 2-DG. At the conclusion of the experiment arterial plasma samples were collected for the determination of hindlimb glucose uptake ( ). After samples were taken at 120 minutes animals were sacrificed and calf and epididymal fat pads were immediately excised, weighed (epididymal fat pads only) and freeze clamped in liquid nitrogen and stored at -80ºC.

6.2.3 Gene expression

Expression of inflammatory markers was performed by two step reverse transcription real-time PCR (RT-q-PCR). RNA was first extracted from skeletal muscle and adipose tissue samples and reverse transcribed using SuperScript III (Invitrogen) to obtain cDNA as described previously in Chapter 2, section 2.3. Real time PCR (qPCR) was performed using SYBR Green (Qiagen) to amplify the target DNA sequence and quantify gene expression of the chosen inflammatory markers (iNOS, TNFα, MCP-1, and EMR1) as described in Chapter 2, section 2.3. Inflammatory gene expression is shown as the relative expression to the housekeeper S9. The gene expression measurements were powered to detect changes of 50% or greater.

6.2.4 Data and statistics

Data is present as means ± SEM and statistical analysis was performed using SigmaStat (Systat Software Inc). Comparisons between groups were made using un-paired Student's t-test and two-way ANOVA. Comparison of time-series measurements in each group was performed by two-way repeated measures ANOVA. When a significant difference of $p<0.05$ was detected, pairwise comparisons by Student-Newman-Keuls test was used to assess treatment differences.

6.3 Results

High fat fed rats that received metformin treatment were significantly lighter in body weight compared to high fat fed rats alone (6.2, A), however this was not reflected in a significant difference in epididyal fat pad weight (Figure 6.2, A).

Metformin treatment in high fat fed rats resulted in significantly improved whole body insulin sensitivity with both glucose infusion rate during hyperinsulinemic euglycaemic clamps significantly improved compared to high fat fed rats without treatment (Figure 6.3). In control animals there was no change in whole body insulin sensitivity between animals that did and did not receive metformin as shown by GIR (Figure 6.4, A). In high fat fed animals metformin significantly improved whole body insulin sensitivity (GIR) compared to high fat fed animals that did not receive metformin. The improvement in GIR of metformin-treated high fat fed rats was also shown to be significant compared to control animals (Figure 6.4, A). Metformin treatment did not alter glucose uptake by the skeletal muscle of control animals (Figure 6.4, B). Metformin did significantly improve skeletal muscle glucose uptake in high fat fed rats compared to high fat fed animals that did not receive metformin, and restored it to that of control animals (6.4, B). Skeletal muscle microvascular perfusion remained unchanged in control diet-fed animals with and without metformin (6.4, C). In high fat fed rats metformin significantly improves microvascular recruitment in the skeletal muscle of high fat fed rats compared to high fat diet alone, and restored microvascular blood flow to that of control animals (6.4, C).

The expression of inflammatory and macrophage markers within adipose tissue were not different between the high fat fed rats which received metformin and those that did not (6.2).

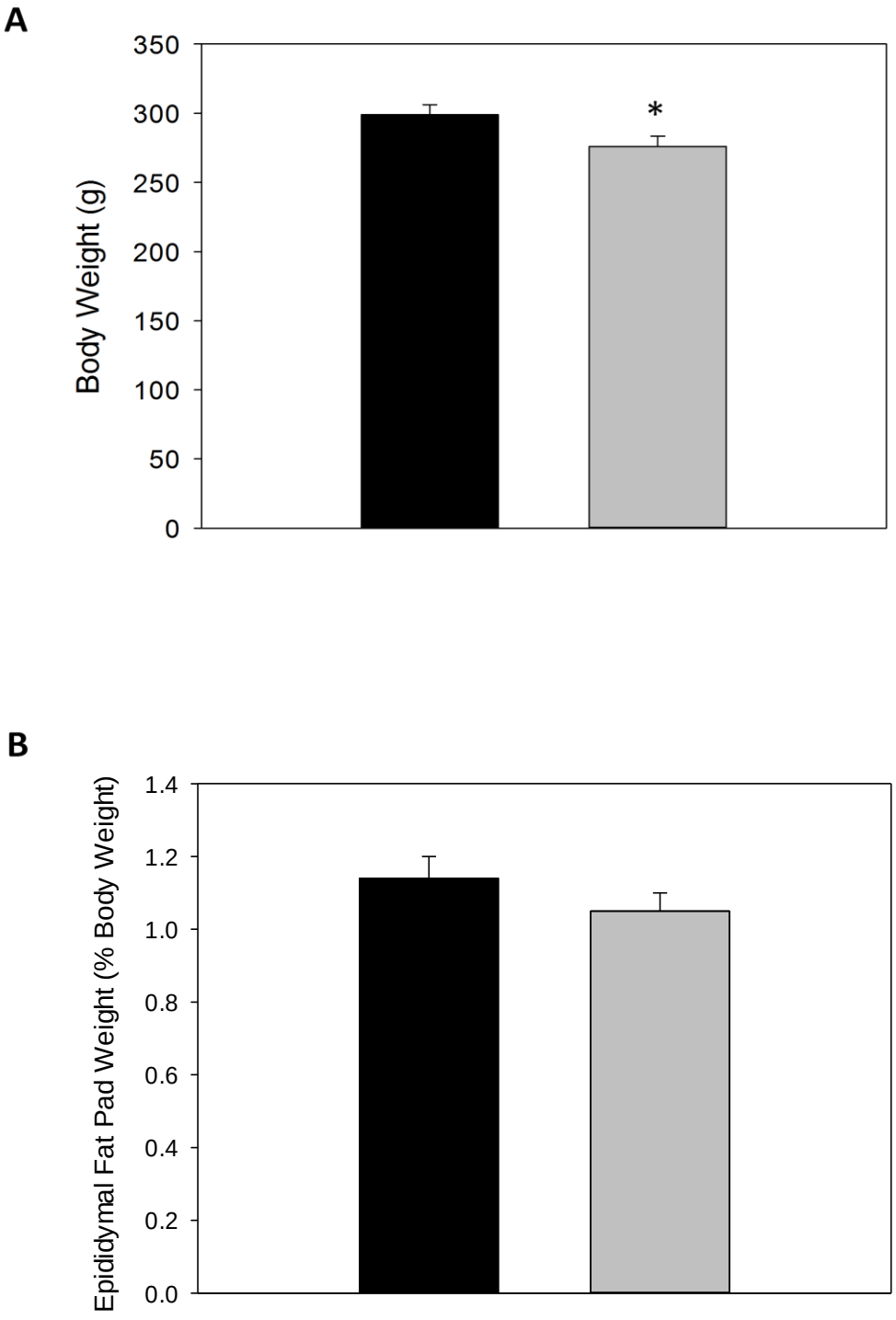

Figure 6.2 – Effect of 4 weeks high fat feeding (41% fat) and metformin treatment on (A) body weight and (B) adiposity in high fat fed rats (■), and high fat fed rats treated with metformin (□). Epididymal fat pads were excised and weighed immediately after removal. Mean wet weight as percentage of body weight is shown for each group ± SEM for n = 25-27 rats. * = $p<0.05$ indicates significant difference by Student's t-test.

Table 6.2 - Effect of metformin treatment on metabolic responses and insulin sensitivity in high fat fed rats.

	HFD	HFD + Met	Sig
Glucose Infusion Rate (mg/min/kg)	18.0 ± 0.1	26.4 ± 0.1	*P < 0.001*
Basal blood glucose (mmol/L)	3.6 ± 0.1	3.8 ± 0.1	NS
Muscle glucose uptake (R'g) (µg/g/min)	15.7 ± 1.1	22.8 ± 1.7	*P < 0.01*
Mean Arterial Pressure (mmHg)	111 ± 2	111 ± 2	NS

Metabolic parameters were measured by performing 10 mU hyperinsulinemic euglycaemic clamps in anaesthetised animals, with glucose infusion rate reflecting whole body insulin sensitivity. Muscle glucose uptake was calculated by 2-deoxy-D-Glucose uptake during final 45 minutes of clamp procedure. Data shows means ± SEM for n = 14-17 rats in each group. Statistical analysis was performed by Student's t-test and GIR analysis was performined by two-way repeated measures ANOVA.

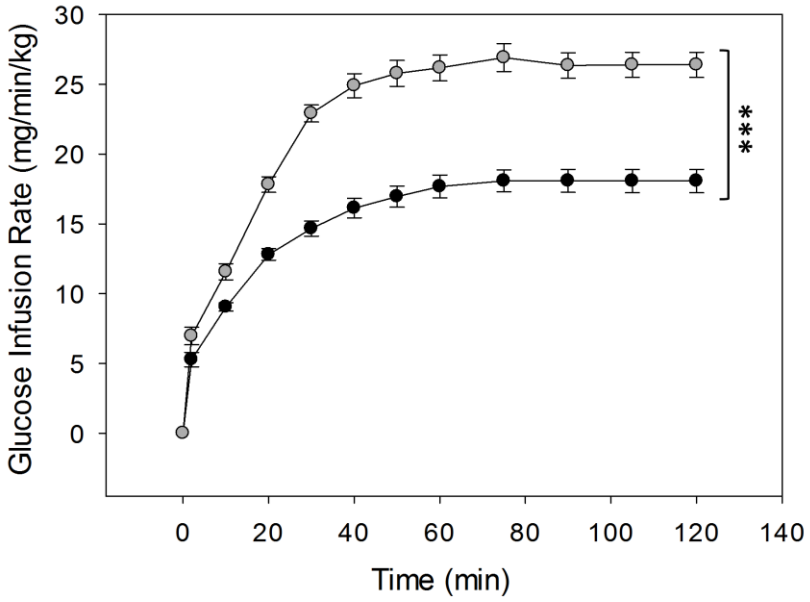

Figure 6.3 – Effect of a 4 week 41% high fat diet and metformin treatment on whole body insulin sensitivity of high fat fed rats (●), and high fat fed rats treated with metformin (○) by hyperinsulinemic euglycaemic clamp. Data shows means ± SEM for n = 14-17 rats in each group. *** = $p < 0.001$ indicates significant difference between high fat diet groups with and without metformin treatment for GIR measurement by two-way repeated measures ANOVA.

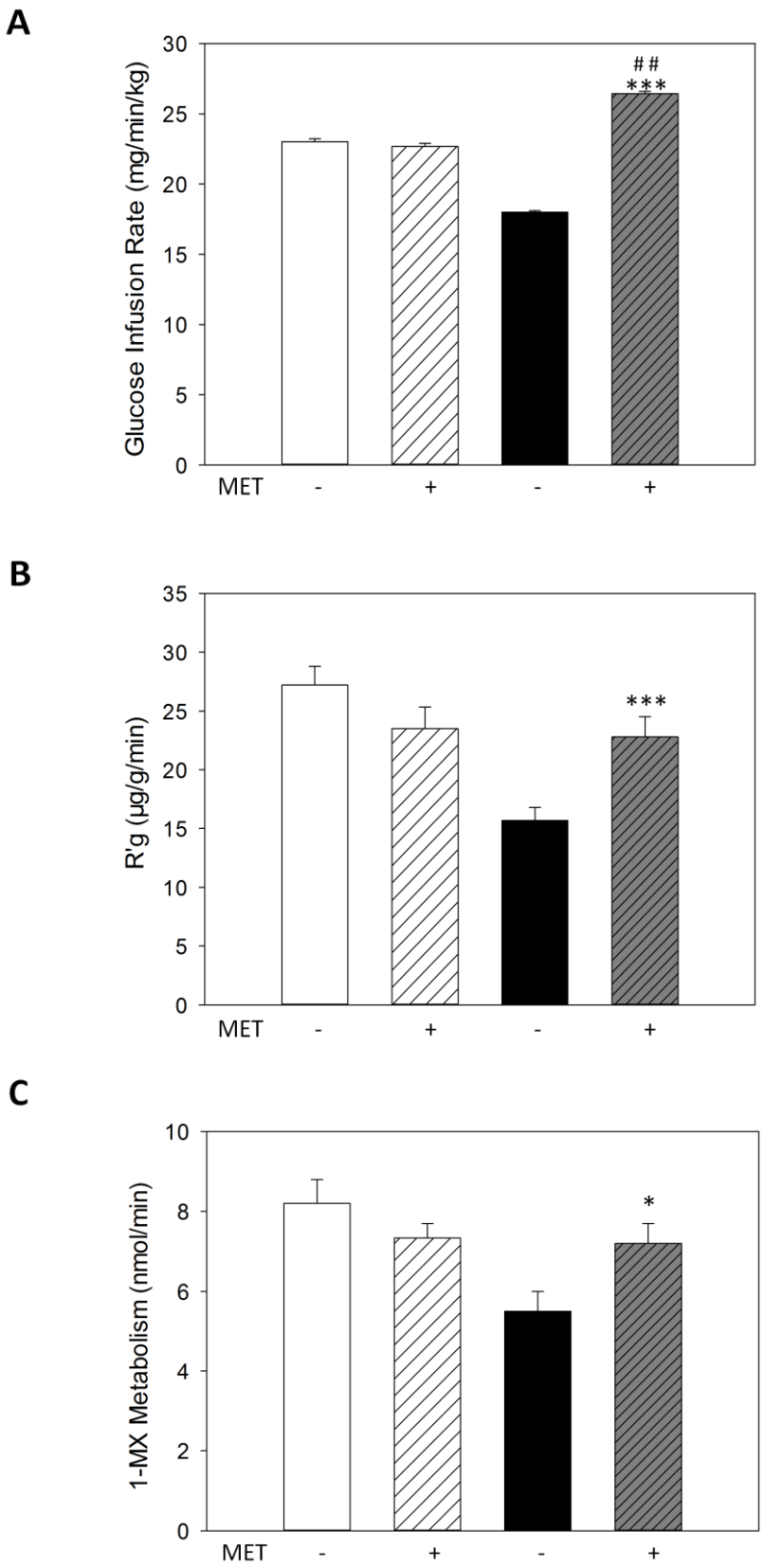

Figure 6.4 – Effect of a 4 week 41% high fat diet and metformin treatment on (A) whole body insulin sensitivity, (B) skeletal muscle glucose uptake, and (C) Microvascular perfusion in control (□) and high fat fed rats (■), with and without metformin treatment. GIR reflects whole body insulin sensitivity during 10 mU hyperinsulinemic euglycaemic clamps. Muscle glucose uptake was calculated by 2-deoxy-D-Glucose uptake during final 45 minutes of clamp procedure, and microvascular perfusion within skeletal muscle was assessed by the metabolism of 1-MX. Data shows means ± SEM for n = 14-17 rats in each group. * = $p<0.05$, ** = $p<0.01$, *** = $p < 0.001$ indicates significant difference within diet groups with and without metformin treatment, ## = $p<0.01$ indicates significant difference between high fat and control rats treated with metformin by two-way ANOVA.

Table 6.3 – Comparison of inflammatory gene expression in insulin sensitive tissues of 4 week high fat diet-fed rats with and without metformin treatment

EPIDIDYMAL FAT			
	HFD	HFD + Met	*Sig*
iNOS	164 ± 36	84 ± 1	*NS*
TNFα	35 ± 3	41 ± 3	*NS*
EMR1	635 ± 70	532 ± 70	*NS*
MCP-1	1490 ± 383	1494 ± 285	*NS*

Table shows relative expression of inflammatory marker mRNA ratio to the housekeeper S9 in Sprague Dawley rats fed a 41% high fat diet for 4 weeks with and without metformin treatment. Inflammatory gene expression was not found to be altered in the visceral adipose tissue of high fat fed rats treated with metformin compared to those without. Data shows means ± SEM for n = 5-6 rats in each group. Statistical analysis was performed by Student's t-test.

6.4 Discussion

Metformin is the drug of choice therapy for the treatment of insulin resistance in humans. This is due to both its effectiveness at controlling blood glucose levels and preventing cardiovascular mortality [255, 268, 271]. This study has confirmed that high fat fed rats treated with metformin have significantly improved whole body insulin sensitivity and muscle glucose uptake compared to high fat diet alone (Table 6.2, Figure 6.3). This study has also confirmed that metformin improves muscle insulin sensitivity in terms of glucose uptake during an insulin clamp. Comparison of GIR of control and high fat fed rats shows that metformin treatment in high fat fed rats not only significantly increases whole body insulin sensitivity compared to high fat diet alone, but it actually restores and significantly improves whole body insulin sensitivity compared to that of control diet-fed animals (Figure 6.4, A). Skeletal muscle glucose uptake in high fat fed animals was also significantly improved and restored to that of control animals when treated with metformin (Figure 6.4, B). Comparison of microvascular perfusion by 1-MX shows that metformin not only significantly improves microvascular blood flow within the skeletal muscle of high fat fed rats, effectively restoring it to that of control-fed animals (Figure 6.4, C). Measurement of inflammatory gene expression within the adipose tissue confirmed that 41% high fat feeding does not induce a significant inflammatory response and this was not altered by metformin treatmed despite its glucoregulatory actions (Table 6.3). These results confirm that the loss of insulin-mediated metabolic and vascular functions are not driven by inflammation and can be restored.

The finding that 4 week metformin treatment can restore microvascular blood flow within insulin resistant muscle to that of controls has not yet been reported elsewhere in the literature. The ability to not only improve but restore microvascular blood flow to that of controls would have direct consequences for muscle glucose uptake due to enhanced delivery of glucose to the myocytes. Metformin's ability to improve capillary blood flow so completely may be why it is so successful at improving skeletal muscle insulin sensitivity as it has been shown that the loss of microvascular blood flow responses is a leading contributor in the development of skeletal muscle insulin resistance [86]. These results confirm the effectiveness of metformin for the treatment of insulin resistance and that it is a drug

treatment that targets multiple aspects of insulin resistance by significantly improving both metabolic and vascular dysfunction.

In conclusion, this study confirms the complete restoration of insulin's metabolic and vascular effects on muscle by metformin treatment, and demonstrates that this action does not require an anti-inflammatory action in adipose tissue. This does not rule out the possibility that in situations where inflammation contributes to insulin resistance, that metformin might have additional benefits for muscle or whole body insulin sensitivity. However, it suggests that metformin's actions in muscle and its vasculature are more likely by opposing the 'lipotoxicity' effects on insulin signalling induced by altered plasma and whole body lipid distribution. It remains to be seen whether there is any additional benefit for muscle insulin sensitivity in a more complex model of insulin resistance, perhaps reflecting human obesity, where inflammation and dyslipidemia may be present together.

CHAPTER 7

DISCUSSION

7.1 Findings and General Discussion

The increasing prevalence of obesity has become a significant problem. This is due to its strong association with a number of health conditions including insulin resistance, Type 2 Diabetes, and cardiovascular disease [16, 18, 20, 116, 141]. Many studies have induced obesity in animal models in order to investigate these conditions and gain a greater understanding as to the mechanisms and actions regulating their induction [86, 138, 176, 181, 193, 210]. Although genetic models of obesity are regularly used [129, 189, 235, 278, 279], diet-induced obesity has been favoured by many as it provides a more accurate representation of the development of obesity and its associated health complications [26, 188, 202, 204, 212, 220]. Dietary interventions to induce obesity have primarily focused on increased fat content. This is largely due to increased dietary fat being seen as a significant contributing factor towards the development of obesity in humans and animals [204, 279, 280]. A number of studies have investigated variable degrees of fat on obesity and metabolic responses in rodent models [26, 86, 138, 180, 200, 209, 215, 281]. In both rats and mice, moderate to high fat feeding within the literature has proven successful at producing significant obesity accompanied by associated insulin resistance, with diets containing a higher fat content or given for longer interventions being shown to produce both a greater degree of adiposity and metabolic impairment [26, 86, 180, 198, 202].

Results presented in Chapters 3 and 4 have shown varied responses to high fat dietary interventions in rats. A 'moderate' high fat diet in which 41% of the calories were derived from fat, produced significant adiposity in rats after a 4 week dietary intervention (Chapter 3). High fat fed rats showed approximately a 50% increase in adipose tissue weight compared to control animals, with this increased adiposity associated with impaired insulin sensitivity of both metabolic and vascular responses. Many obesogenic studies in both mice and rats favour diets with considerably higher fat contents in which more than half of the total calories are derived from fat [26, 72, 86, 176, 198, 200, 201, 203, 209, 282]. Diets in which approximately 58% of the calories were derived from fat produced significant adiposity in rats after a 4 week intervention which was approximately 50% greater than that of control animals. Surprisingly, this adiposity was not associated with the same metabolic impairments present in rats on the 41% fat diet (Chapter 4) or those reported in the literature [86, 176]. An

even more surprising finding was that extending this dietary intervention to 12 weeks of high fat feeding did not produce a phenotype typical of diet-induced obesity in our rat model. In fact this 12 week high fat intervention showed that adiposity seen following 4 week feeding was not maintained with longer dietary intervention. Therefore this higher fat diet only produced transient adiposity which was not maintained long-term. Despite its reported success within the literature [26, 86, 117, 120, 176, 177, 203], a 58% high fat diet was shown to be ineffective at inducing an obese and insulin resistant phenotype in our rat model (Chapter 4).

Results from high fat feeding interventions in chapters 3 and 4 have shown that although some high fat diets are successful at producing an obese and insulin resistant phenotype in rats, they do not necessarily deliver these responses consistently or maintain them over long interventions. From the results obtained, it is clear that from the two high fat diets used, the diet with the lower fat content (41% fat) was more effective than the higher fat diet (58% fat) at producing obesity (75% versus 50% increase) and insulin resistance in rats. Although this result was initially surprising due to the reported success of high fat feeding for increasing adiposity within the literature, it raised the question of diet palatability playing a key role in inducing hyperphagia and thus obesity in rat models. The high fat diet may not be palatable enough to produce and maintain a significant degree of obesity in rats as dietary intervention lengthens. This explains why adiposity was transient with the 58% high fat diet and only rats given short-term interventions showed significant adipose tissue accumulation. Therefore use of diets high in purely fat has shown that alternative interventions to induce diet-induced obesity in rat models should be considered. A cafeteria-style diet based on a variety of highly palatable snack-food items has not only shown to produce significant hyperphagia and weight gain in rat models, but also provides a more realistic representation of poor dietary health often present in obese humans [188, 220, 222, 227, 228].

As shown in Chapter 5, the use of this highly variable cafeteria-style diet based on palatable and calorie dense snack food items produced excessive adiposity and significant insulin resistance after a 4 week intervention. The degree of adiposity produced with this diet equated to approximately a 120% increase in adipose tissue accumulation compared to controls. Long-term intervention showed that adiposity remained significantly elevated and

insulin sensitivity was still impaired after 12 weeks. Direct comparison of these results to those obtained with 58% high fat feeding (Chapter 4) over both short and long-term interventions show that the use of this palatable and variable cafeteria-style diet is superior at inducing an obese and insulin-resistant phenotype in rats compared to a diet high in fat alone. Comparison of adiposity after a 4 week intervention shows that cafeteria diet-fed rats possessed approximately an 80% increase in adipose tissue weight compared to the adiposity of 58% high fat-fed rats, whilst after 12 weeks cafeteria diet rats possessed a 150% increase in adiposity compared to matched high fat fed rats.

Therefore these results show that both diet variability and palatability are important components to consider for the induction of diet-induced obesity in rats, and this conclusion is consistent with studies conducted by Sampey [188], Prats [232], Shafat [227], Rogers [222], and Martire [220] using similar dietary interventions. However this thesis adds to the current research due to its ability to directly compare the degree of obesity and insulin resistance obtained with a cafeteria-style diet to that obtained with commercial high fat feeding over both short and long-term time-points in rats.

Obesity has frequently been associated with inflammation within the literature, and this obesity-induced inflammation has regularly been reported to contribute towards the metabolic dysfunction present in insulin resistant conditions [18, 19, 100, 118, 148, 181]. However despite this association, the mechanisms surrounding its induction and ability to impair insulin sensitivity is not well understood. Much of the work in regards to obesity-induced inflammation has been conducted in high fat fed mouse models [26, 27, 117, 120, 123, 124, 187] with similar studies in rats [185, 188, 210] outnumbered by comparison. Reasons for this preference have not been addressed within the literature, however rat models allow for more thorough examination of physiological systems such as vascular responses within skeletal muscle [23, 32, 86, 87]. As a result of the strong association between obesity and insulin resistance, the inflammatory markers chosen in this thesis included those that had shown the ability to alter insulin-mediated responses within the vasculature and skeletal muscle [72, 122, 123, 126, 127, 129, 142, 143, 187, 253].

Diet-induced obesity in mice has shown that a number of inflammatory and macrophage markers are significantly increased within visceral adipose tissue within 1 week of high fat feeding, with some markers significantly expressed within 3 days. However a greater significant inflammatory response was seen in the adipose tissue, liver, and skeletal muscle after long-term intervention consisting of 16 weeks in which obesity was the most pronounced [26]. Some of these markers include TNFα, iNOS, MCP-1, and EMR-1 which were the inflammatory factors selected to be investigated in this thesis. Significant inflammatory responses within the literature have been reported as large fold changes such as 2-3 fold increases gene expression within tissues, with some studies reporting expressions that are as high as 10 or 20 times higher [26, 71, 124].

From the dietary studies conducted in this thesis there was a notable absence of inflammatory expression in both models of high fat feeding regardless of obesity and insulin resistant status. However what was especially surprising was that despite the excessive adiposity present in cafeteria diet-fed rats, inflammatory factors were not found to be significantly expressed at either 4 or 12 week time-points. What is suggested from this study is that obesity and insulin resistance can occur independently of inflammation, and that although obesity-induced inflammation may in turn influence insulin-mediated responses if induced, it may not play an important role in its initial induction. Nor does improvement to insulin sensitivity alter the expression of inflammatory factors within tissues. Although further confirmation and examination of inflammation and insulin resistance in diet-induced obesity is needed, regular reporting of its association within obese and insulin resistant conditions should be reconsidered if additional findings support this conclusion.

The examination of other tissues such as liver could provide further evidence of inflammatory status with dietary interventions. Studies conducted by Lee [26] and Sampey [188] have previously investigated dietary induced liver inflammation in mice and rats respectively. Both studies report significant inflammation with increased macrophage infiltration and expression of pro-inflammatory markers such as TNF-α, IL-6, IL-1β, iNOS and MCP-1 in the liver and adipose tissue of 16 week high fat fed mice [26] and 15 week cafeteria diet-fed rats [188]. However as there was a significant lack of inflammatory response within the adipose tissue of 12 week high fat and cafeteria diet-fed rats from the

studies conducted in this thesis, the presence of significant liver inflammation is therefore unlikely in these animals, however liver tissue should be considered in further experiments. In addition to liver, further examination of adipose tissue depots may also provide additional insight into inflammatory expression in obesity. There has been notable interest in the inflammatory expression within fat depots surrounding blood vessels (perivascular adipose tissue) within the literature [283-288]. Perivascular adipose tissue has been shown to produce a wide variety of adipokines, with a number of studies having shown significant expression of proinflammatory cytokines and chemokines within this fat depot [283, 284, 287]. As previously discussed within the introduction of this thesis, a number of proinflammatory factors have been shown to intervene with the highly coordinated actions of the vasculature and alter vascular responses [72, 126-128, 143, 160, 161]. As a result fat depots both within and surrounding the heart have been of interest to those investigating the links between inflammation and heart disease [285, 287]. Cardiovascular disease is highly prevalent in obese and insulin resistant individuals [12, 100, 141, 289] and as previously discussed vascular dysfunction, especially within the microvasculature, is significant in the development of impaired skeletal muscle insulin sensitivity [16, 23, 32, 86, 138]. Therefore there have been a number of studies that have investigated the relationship between proinflammatory perivascular adipose tissue, vascular function, and insulin sensitivity with many suggesting that this fat depot may contribute more directly to the pathogenesis of the metabolic syndrome [285, 287, 288, 290].

In mouse studies of obesity-associated inflammation the degree of adiposity is often reported to be 3 or more times higher than that of control animals. This degree of adiposity is comparable to that obtained in the 12 week cafeteria diet-fed rats, however these rats did not show a significant inflammatory response. Therefore the lack of inflammatory expression despite significant and comparable adiposity is intriguing. A range of inflammatory factors have be shown to be significantly expressed within both the adipose tissue and skeletal muscle of obese mice [26, 27, 117], however interestingly this response does not translate across in rat models of diet-induced obesity. This raises the possibility that rats are much more resistant to the development of inflammation and may possess mechanisms to protect against developing obesity-associated inflammation in particular. As the results obtained from the cafeteria-diet study show, both short and long-term interventions produced excessive obesity in rats however there was no increased expression of inflammatory factors

within the adipose tissue of these animals compared to controls (Chapter 6). Therefore it is possible that straightforward obesity alone is not enough to trigger an inflammatory response and that other factors in association to obesity may be required for this to occur or to initiate an inflammatory outcome.

7.2 Implications

Both insulin resistance and inflammation are complex disease states that involve a number of highly controlled signalling pathways [18, 19, 43, 53, 56, 96, 100, 164, 199, 291-295]. There has been much research in investigating the involvement of numerous cells and proteins on altering these pathways in the development of obesity-induced inflammation [112, 113, 156, 247, 295-299]; however despite this a number of specific mechanisms such as when and how inflammation is induced in diet-induced obesity, and a detailed time-course of how its induction impacts on vascular and muscular insulin-mediated responses, is still not fully understood [26, 117, 148, 198, 210]. What is clear from previous research is that the induction of inflammatory signalling, especially within diet-induced obesity, may be reliant on a number of other signals and responses to occur before inflammation can be initiated. Due to the strong association between obesity and inflammation, the adipose tissue has been highlighted as an important site in which the regulation of inflammatory expression is conducted [19, 27, 164, 165, 300]. The view that adipose tissue is solely a site for energy storage is now outdated. Adipose tissue is now recognised as a complex and active organ capable of multiple functions and that, in addition to its primary role in lipid storage, also contributes towards endocrine function [163, 165]. The adipose tissue is known to secrete a number of hormones including leptin and adiponectin which are important for metabolic and energy homeostasis [163-165]. In addition to this the adipose tissue also secretes or induces a number of inflammatory factors including cytokines and macrophage markers. However the exact mechanisms and pathways by which this occurs are still not fully understood.

Due to its primary role for energy storage, the adipose tissue is required to respond rapidly to changes in nutrient availability and react accordingly [166]. In times of excess nutrient availability, adipocytes are required to undergo hypertrophy and at times hyperplasia to

accommodate for additional energy storage. Therefore the tissue requires mechanisms in place that allow for regular and ongoing remodelling of the tissue in order to meet these demands. At times in which nutrient availability and storage requirements are regularly needed in excess (such as during high fat feeding or diet-induced obesity), this process of hypertrophy and tissue remodelling would be required at a much higher rate [166].

Adipose tissue regulation and remodelling, especially during expansion in obesity, has gained interest in regards to the tissue's inflammatory status and associated health implications and has thus become an important topic for discussion [165, 168, 301-303]. Adipose tissue goes through an ongoing process of tissue remodelling and expansion during the development of obesity or rapid lipid storage in order to meet with storage demands. However it has been shown that adipose tissue can undergo both healthy and unhealthy tissue expansion. Unhealthy tissue expansion has been linked to pathological and metabolic dysfunction often reported in obese and diseased states, such as inflammation [165]. The exact reasons and key influencing factors for why adipose tissue may expand in healthy or unhealthy ways is not fully understood and requires further in-depth examination, however some mechanisms have been hypothesised.

During early or short-term lipid storage adipose tissue will undergo expansion to meet with storage demands, however if storage demand increases chronically the tissue can undergo remodelling and hyperplasia [165]. Under healthy expansion conditions angiogenesis may also occur to meet and provide the tissue with a sufficient blood supply and oxygen delivery [166]. However excessive lipid storage and rapid expansion of the adipose tissue in a short amount of time may result in significant hypertrophy of adipocytes without additional remodelling which includes adequate hyperplasia and angiogenesis. This can result in insufficient vascular or microvascular branching to allow adequate blood flow and oxygen delivery to the adipocytes. Excessive hypertrophy would also reduce the diffusion distance and oxygen availability within the adipocytes themselves. During significant hypertrophy, adipocytes may reach a degree of expansion in which the diffusion of oxygen into the cell is limited. Adipocytes have been found to reach sizes up to 150-200 μm in diameter during obesity, which is larger than the average diffusion distance of oxygen across tissues [166, 304, 305]. Both of insufficient blood supply and poor oxygen diffusion can result in a

hypoxic environment occurring within adipocytes, which in turn increases the expression of Hypoxia Inducible Factor 1α (HIF-1α) [167, 168, 303, 305]. HIF-1α has been identified an early upstream initiator of inflammatory responses, and thus this hypoxic environment and activation of HIF-1α within the adipose cell can activate inflammatory factors including the induction of macrophages to occur within the tissue [165, 166, 168]. Some adipocytes may also become so poorly oxygenated that they become necrotic which in turn has been shown to activate an increased inflammatory responses locally within the tissue [166, 168, 170, 171]. Therefore the development of hypoxia within the adipose tissue is potentially a key factor underlying the induction of inflammatory responses within the tissue itself, leading to the reported association of inflammation with obese conditions [305]. Constant remodelling and stress within the adipose tissue due to the need to meet storage demands may also result in fibrosis which can impair tissue expansion and be another contributing factor to an unhealthy adipose tissue environment [166, 168, 306]. Fibrosis within the adipose tissue has also been highlighted, in addition to hypoxia, as a key factor important for the initiation of local inflammation within the tissue [166].

Despite this, the regulation of adipose tissue expansion is still not greatly understood and the mechanisms managing these responses as to whether dysfunctional expansion will occur are still unknown. However, adipose tissue expansion has gained interest due to the potential health implications that unhealthy expansion may have both within the tissue and systemically [165, 168, 301-303]. Because of this, dysfunctional adipose tissue expansion and hypoxia have been identified as important factors involved in the initiation and development of obesity-associated inflammation regularly reported within the literature. Therefore healthy expansion within the adipose tissue may provide an explanation for the lack of inflammatory response in our rat models of diet-induced obesity. However further confirmation is needed in order to draw a reliable conclusion. Histological assessment of the adipose tissue, especially in regards to adipocyte size, macrophage content, and HIF-1α expression, would provide additional information as to the health and inflammatory status of the tissue and inclusion of this measurement would have provided considerable weight towards the conclusions of this thesis. Further investigation into HIF-1α and adipose tissue expansion and blood flow would be beneficial for future studies into diet-induced inflammation as there is potential possibility that the inflammatory status within the adipose tissue is variable during periods of expansion, and that adipose tissue inflammation may be

able to directly affect other responses such as vascular signalling during this expansion process if unhealthy adipose tissue expansion occurs.

7.3 Limitations

The main limitation and weakness of the current thesis is the measurement of inflammation within this study. A more detailed and comprehensive assessment of inflammation would provide further evidence to support the final conclusions of this thesis. In the gene expression studies only four inflammatory markers were investigated in this thesis which does result in limitations within the study and thus its conclusions. Future experiments should be expanded to investigate a wider range of inflammatory markers which have also been association with obesity and insulin resistance such as IL-6 [18, 118, 123, 124, 130], IL-10 [130], NF-$_K\beta$ [18, 112], HIF-1α [168, 200, 303, 305, 307] and a greater number of macrophage markers such as CD11c [208] and CD68 [123, 308]. In addition, the inflammatory gene expression studies could be further strengthened by the use of positive controls. The presence of a controlled inflammatory response would have allowed a direct comparison of the degree of inflammation obtained and provided additional support towards the conclusions of this thesis. Therefore the lack of positive inflammatory controls is currently a weakness within the study and should be included in any further experiments. Another point to note is that although the gene expression studies were powered to detect a change of 50% or greater within the current thesis, the statistical power and sample sizes for the gene expression studies should be reviewed in light of the data obtained. There is the potential that these experiments may be incorrectly powered and thus limit the effectiveness of identifying true significant inflammatory changes and influencing the conclusions of this thesis. The examination of the full cohort of animals instead of a subset (especially within Chapter 3) should be considered in any future experiments. Re-evaluating the statistical power and sample size needed to detect significant changes would be important for any further examination of inflammatory gene expression.

Additional methods of evaluating inflammatory responses such as flow cytometry, histology, and investigating other fat depots and tissue sources are all measurements which would also

add further weight to the gene expression studies. The use of flow cytometry would be a powerful technique to provide additional data as to both the presence and subset of immune cells, including the presence of circulating inflammatory cells within the plasma. In addition, the use of histology and immunofluorescence analysis would allow for direct examination of the infiltration of inflammatory cells within tissues such as skeletal muscle and adipose tissue. Histology analysis would also allow for the degree of lipid accumulation, hypertrophy, and hyperplasia to be examined within the adipose tissue and provide additional insight into the health and expansion of the tissue itself.

As well as including additional techniques to assess inflammation, expanding the sites and tissue samples analysed would also add further evidence to this thesis. The use of the epididymal fat pad depot as the site for adipose tissue inflammation is not entirely a poor choice as epididymal fat pad has been routinely examined by others within the literature due to its visceral nature, ease of access for removal, and significant lipid accumulation [26, 86, 138, 176, 188]. Visceral fat has been strongly linked to metabolic disease and increased inflammatory expression within the literature [118, 300, 309-312], however it is important to note that investigating other fat and tissue depots could provide additional insight into inflammatory expression in obesity. Therefore the use of only skeletal muscle and visceral adipose tissue is currently a weakness within the study and other tissue sources, such as liver and other fat depots such as perivascular adipose tissue, which have also shown increased inflammatory expression linked to obesity and impaired insulin sensitivity [26, 142, 154, 188, 285, 287, 288, 290], should be investigated in future experiments.

Providing other measurements in addition to body weight and adiposity could offer further insight into the body composition and potential overall health of the animals given different dietary interventions, and thus the effectiveness of these dietary interventions on producing a phenotype more typical to that of human obesity. Limitations of only body weight and epididymal fat pad measurements also do not take in to account the lean mass or the natural growth changes that may occur between animals within the studies. Despite the use of age-matched controls within all experiments, this may be of importance as the experiments have been conducted in relatively young animals. Although investigating the effects of dietary intervention and high fat feeding within younger animals is relevant due to the increasing rise

of childhood obesity and insulin resistance [8, 10, 313, 314], dietary intervention in young animals may be a potential confounding factor in regards to the true degree of weight gain and adiposity that is obtained due to growth that occurs within this age range. Performing comparative studies in older, more established animals should be considered for future studies and would provide additional information in regards to adiposity and the degree of inflammation and impaired insulin sensitivity obtained with dietary intervention.

In addition to the studies being conducted in younger animals only male rats were chosen to study within these experiments. The majority of our research group's characterisation in models and measurement of vascular responses has been performed in male rats [23, 86, 126, 138, 143, 176], therefore conducting these experiments in all-male animals allows for a direct comparison between studies previously conducted within our group. In addition, the experiments within this thesis attempted to gain a greater understanding of underlying mechanisms of action between obesity-induced inflammation and insulin resistance, so at this stage the inclusion of both sexes currently adds additional variability. The discovery of a significant association or link would be preferable before the study is broadened to include more additional variables, however it is possible for sex-related differences and responses to occur and the inclusion of both sexes within experimental studies is valid and should be considered in future investigations.

There were also notable difficulties with the use of high fat diets within the experiments of this thesis. Both the 41% and 58% high fat diets have been used previously in studies within our research group with both diets proving to be successful at impairing insulin sensitivity after similar dietary interventions [86, 138, 176, 180]. Unfortunately the results from previous studies using the 58% high fat diet [86, 176] could not be replicated within this thesis and in fact produced problematic results in regards to adiposity and insulin sensitivity with long-term dietary use. As the use of the 58% high fat diet was proving difficult it was decided to abandon commercial laboratory diets in favour for a cafeteria-style diet consisting of high caloric and palatable snack food items. However it would be important to further investigate the 58% high fat diet in more detail including monitoring food intake to investigate whether diet palatability and caloric intake can explain the lack of adiposity in animals fed this diet over longer interventions. Without this measurement it can only be

proposed that calorie intake and food consumption was lower with this diet and that palatability of the diet may be a significant contributing factor. Inclusion of the 41% high fat diet in an independent cohort of rats during this investigation may also provide a useful comparison, especially in regards to the degree of adiposity and impaired insulin sensitivity that is obtained.

Another weakness within this thesis is that microvascular perfusion was not measured in all rat experiments compared to skeletal muscle insulin sensitivity. Microvascular perfusion has previously been examined by our research group with impaired microvascular blood flow found to be an early defect in the development of impaired insulin sensitivity [32, 86, 138]. Therefore the absence of skeletal muscle insulin resistance in short and long-term high fat fed rats in Chapter 4 implies that there is no additional vascular defect also present, however inclusion of this measurement would have provided confirmation. Microvascular perfusion was also not measured in cafeteria diet-fed animals which did show significant impairment to skeletal muscle insulin sensitivity and is thus currently a limitation within the study as this measurement would have provided a more comprehensive view of the dysfunction and impaired insulin sensitivity of these animals, comparable to the high fat studies conducted in Chapters 3 and 6. Also of note is that although insulin-mediated glucose uptake in skeletal muscle has been a routine measurement, the inclusion of insulin-mediated glucose uptake within adipose tissue could have provided additional evidence of adipose tissue insulin sensitivity. This would have provided additional support towards the conclusions of this thesis, especially if the adipose tissue was found to show significant impairment to insulin sensitivity without increased inflammatory expression.

7.4 Conclusions

Results from this thesis have shown a number of findings in regards to diet-induced obesity and obesity-associated inflammation in rats. Firstly, dietary interventions to generate diet-induced obesity should be considered carefully. Use of commercial high fat diets in rats has shown variable results in regards to producing an obese and insulin resistant phenotype. Diet

variation and palatability have been shown to be important factors to consider in regards to generating significantly overt diet-induced obesity in rats. Secondly, inflammation does not appear to be an important contributing factor in regards to the development of obesity and insulin resistance in rats as obesity and insulin resistance can develop, and be restored, without the presence of increased inflammatory expression. Results obtained in this thesis have indicated that obesity-associated inflammation may not be present in all states of obesity and insulin resistance, and that these conditions may occur without inflammation present, however further assessment of inflammatory expression in diet-induced obesity is required in order to support this conclusion and make further claims. Although inflammatory factors may contribute to impaired insulin sensitivity when induced, it is suggested in the current study that they may not be key factors in driving an insulin resistant response *in vivo*. The lack of significant inflammatory expression despite overt obesity suggests a further hypothesis that rats may possess protective mechanisms preventing inflammatory induction in response to significant obesity. As such, adipose tissue expansion has been highlighted as a potential mechanism that may regulate inflammatory induction in response to diet-induced obesity, however further validation of this theory is required. The mechanisms surrounding healthy and dysfunctional expansion in addition to inflammatory induction are still not well understood and also require further research.

CHAPTER 8

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